

**The tonoplast proton-translocating ATPase from
the crassulacean acid metabolism plant
*Kalanchoë daigremontiana***

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Declaration

The work presented in this thesis is my own and was carried out while a member of a research group. Some of the results presented herein have already been published. The work was directed and supervised by Dr. D.K. Apps and Dr. J.A.C. Smith.

The thesis presented here has been composed by myself.

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Abstract

A rapid procedure was developed for the purification and reconstitution into proteoliposomes of the tonoplast H^+ -translocating ATPase from the Crassulacean acid metabolism (CAM) plant *Kalanchoë daigremontiana*. It involved the fractionation of crude tonoplast membranes with the detergent Triton X-114, resolubilization of the ATPase with octyl glucoside in the presence of an optimized lipid mixture and formation of liposomes on removal of detergent by gel filtration. The enzyme could be further purified by sedimentation through glycerol gradients. It contained polypeptides of apparent molecular mass 72, 57, 48, 42, 39, 33 and 16 kDa; the smallest of these was labelled by [^{14}C]-dicyclohexylcarbodiimide. There was no evidence for the presence of any large subunits. In these proteoliposomes, ATP hydrolysis and H^+ -translocation were measured independently, by a coupled enzyme assay and by quenching of the fluorescence of a permeant weak base, respectively.

The kinetic properties of the reconstituted plant ATPase were studied in detail. Rate equations derived from theoretical models of the enzyme's behaviour were fitted to experimental data by weighted non-linear regression, using a computer program that calculated the kinetic parameters that accorded to the optimal fit. The dependence of the rate of H^+ -translocation on the concentration of MgATP was well fitted by the Michaelis equation, with a K_m value about 30 μM . ATP could be replaced by dATP, ITP, GTP, UTP or CTP and Mg^{2+} by Mn^{2+} or Ca^{2+} ; kinetic parameters for these substrates were determined. In contrast hydrolysis of MgATP showed complex kinetics which suggested either negative cooperativity between nucleotide-binding sites, or the presence of two non-interacting catalytic sites. Both the hydrolytic and the H^+ -translocating activities of the proteoliposomes were inhibited by nitrate, though not in parallel, the latter activity being more sensitive. Both activities were inhibited in parallel by bafilomycin A_1 , which did not produce complete inhibition; the bafilomycin-insensitive component had complex ATPase kinetics similar to those of the uninhibited enzyme. ADP behaved as an allosteric inhibitor of the ATPase, inducing apparent cooperativity in saturation with MgATP, together with a reduction in V_{max} .

By fitting the experimental data to the concerted allosteric model proposed by Monod *et al.* (1965) a theoretical model was proposed in which the complex kinetics were due to the interaction between two substrate binding sites and a single inhibitor-binding site.

Antibodies raised against specific subunits of the tonoplast ATPase were shown to cross-react with V-type ATPases from different species. Immunoblotting of the plant antibodies against the V-type ATPase purified from several bovine tissues has suggested the existence of isoforms of this particular enzyme.

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Abbreviations

ACMA	9-amino-6-chloro-methoxyacridine
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
BzATP	3-O-(4-benzoyl)benzoyladenosine 5'-triphosphate
CAM	crassulacean acid metabolism
CDP	cytidine 5'-diphosphate
CTC	copper-tartrate-carbonate
CTP	cytidine 5'-triphosphate
C ₁₂ E ₉	polyoxyethylene 9-lauryl ether
dATP	2'-deoxyadenosine 5'-triphosphate
DCCD	<i>N,N'</i> -dicyclohexylcarbodiimide
DES	diethylstilbestrol
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β -amino-ethyl ether) <i>N,N,N',N'</i> -tetraacetic acid
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
Hepes	<i>N</i> -(2-hydroxymethyl)piperazine- <i>N'</i> -(2-ethanesulfonic acid)
IDP	inosine 5'-diphosphate
ITP	inosine 5'-triphosphate
kDa	kilodaltons
MEGA-8	octanoyl- <i>N</i> -methylglucamide

NAD ⁺	nicotinamide adenine dinucleotide
NADP ⁺	nicotinamide adenine dinucleotide phosphate
Nbf-Cl	7-chloro-4-nitrobenzofurazan
NEM	<i>N</i> -ethylmaleimide
Oxonol VI	bis(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol
PC	phosphatidylcholine
PEP	phospho <i>eno</i> lpyruvate
PMSF	Phenylmethylsulfonyl Fluoride
PS	phosphatidylserine
Quinacrine	6-chloro-9- $\{[4-(diethylamino)-1-methylbutyl]amino\}$ -2-methoxyacridine
SDS	sodium dodecyl sulphate
TEMED	<i>N,N,N',N'</i> -tetramethylethylenediamine
TID	3-(trifluoromethyl)-3-(iodophenyl)diazirine
TLCK	N α -p-Tosyl-L-lysine Chloromethyl Ketone
TPCK	N-Tosyl-L-phenylalanine Chloromethyl Ketone
Tricine	<i>N</i> -[2-Hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine
Tris	tris(hydroxymethyl)amino-methane
Tween 20	poly(oxyethylenesorbitan) monolaurate
UDP	uridine 5'-diphosphate
UTP	uridine 5'-triphosphate

CHAPTER 1

1. Introduction

1.1. Crassulacean acid metabolism

Many succulent plants exhibit a form of photosynthetic carbon assimilation known as crassulacean acid metabolism (CAM) (Osmond, 1978; Winter, 1985). This is characterized by the nocturnal fixation of atmospheric carbon dioxide into malic acid, which accumulates to high concentrations in the vacuoles of photosynthetic cells (Lüttge *et al.*, 1982). During the subsequent light period this malic acid is decarboxylated and the CO₂ released is refixed by the photosynthetic carbon reduction cycle. CAM along with the C₃ and C₄ photosynthetic pathways are the three major types of photosynthetic carbon metabolism in higher plants. All three pathways utilise the carbon-assimilating enzyme ribulose-1,5-bisphosphate carboxylase (Rubisco: EC 4.1.1.39) to fix CO₂ in the Calvin cycle. Both the C₄ pathway and CAM are ancillary to the C₃ pathway, involving the prior assimilation of CO₂ into C₄ dicarboxylic acids. In C₄ plants, CO₂ is first fixed in the mesophyll as oxaloacetate which is rapidly converted to malate. The malate is then transported to bundle-sheath cells where the CO₂ is released and incorporated into the Calvin Cycle. However, in CAM plants CO₂ is fixed into malic acid at night and stored in the vacuole. The following day the malic acid is decarboxylated and the CO₂ released is incorporated into the Calvin cycle within the same cell. Thus in CAM plants there is a temporal rather than a spatial separation of the uptake of atmospheric CO₂ from the light-dependent assimilation of CO₂.

CAM plants are largely dependent upon the nocturnal accumulation of carbon for their photosynthesis because their stomata are closed during the day in order to retard water loss. Stomatal opening in the dark reduces

transpirational water loss because of the reduced water vapour pressure difference between the leaf and the atmosphere. This increases the plant's water use efficiency i.e. the amount of carbon gained related to water lost. This is clearly an advantage in arid environments which have high light intensity and restricted water availability. It is in these extreme habitats that many CAM species such as the cacti (Cactaceae) and the stonecrops (Crassulaceae) are to be found.

Apart from the nocturnal fixation of CO_2 , the other major feature of CAM is that, because of the nocturnal accumulation of malic acid in the vacuole there is an increase in the intravacuolar osmotic pressure allowing the acquisition and retention of water during the critical night-time period when the stomata are open. Thus by maintaining high osmotically active solute levels at night, CAM plants are further able to reduce their nocturnal water loss.

1.1.1. The metabolic pathway of CAM

The CAM pathway can be divided into two distinct phases: those processes which take place in the dark, and those occurring during the day. These processes are outlined below and summarised in Fig. 1.1.

1.1.1.1. The processes of the dark period

(a) *CO₂ fixation and malic acid synthesis.* The key reaction in CAM is the nocturnal fixation of CO_2 into malic acid. This involves the β -carboxylation of phosphoenolpyruvate (PEP) by PEP carboxylase (EC 4.1.1.31) to form oxaloacetate. Malate dehydrogenase (EC 1.1.1.38) then catalyses the reduction of oxaloacetate to malate.

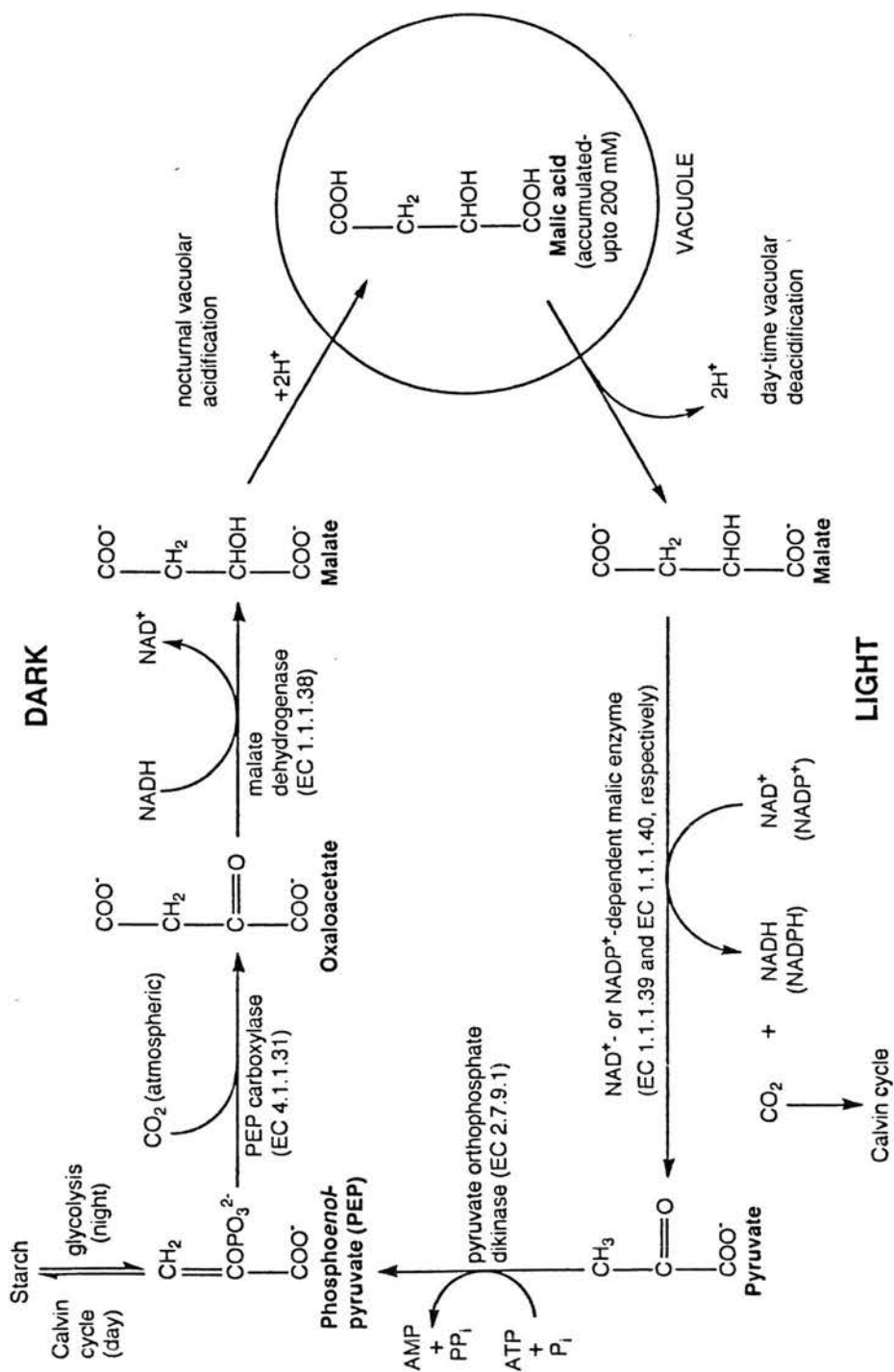


Fig. 1.1. The Crassulacean acid metabolism pathway. At night CO_2 is assimilated into malic acid which is accumulated to high concentrations in the vacuole. During the following daylight period the malic acid is decarboxylated and the CO_2 released is refixed via the Calvin cycle.

(b) *Carbohydrate metabolism in the dark.* In order to provide enough PEP for CO₂ fixation, a considerable amount of stored carbohydrate (starch and glucan) is metabolised at night. Starch is mobilised by one of two routes: either by phosphorylase (EC 2.4.1.1) or by α -amylase (EC 3.2.1.1). The glucose released enters the glycolytic pathway to provide the PEP for CAM.

(c) *The storage of malic acid.* The malate synthesised via dark CO₂ fixation is transported from the cytoplasm to the central vacuole of photosynthetic cells, where it is stored as malic acid. The accumulation of malate in the vacuole and the concomitant decrease in vacuolar pH can be readily observed from measurements of malate concentration and pH in leaf sap. These values can be taken to be the true values for total vacuolar malate concentration and vacuolar pH, since the cytoplasmic volume is very small, i.e. only 0.5 to 1.0% of total cell volume in *Kalanchoë daigremontiana* (Steudle *et al.*, 1980). The concentration of the various charged species H₂malate, Hmalate¹⁻ and malate²⁻ can be calculated from the pH and the pKs of the two carboxyl groups of malate, pK₁ = 3.54 and pK₂ = 5.12. In *K. daigremontiana* the vacuolar malate concentration and pH, at the end of the dark period, were found to be 200 mM and 4.1, respectively (Lüttge *et al.*, 1982). This means that Hmalate¹⁻ is the predominant ionic species in the vacuole at the end of the dark phase. The counter-ions to malate transport have been shown to be provided entirely by H⁺, with 2H⁺ accumulated per malate (Lüttge *et al.*, 1982). However, the experimental values for vacuolar malate concentration and pH at the end of the dark period (quoted above) indicate that the counter-ions to malate cannot be provided by H⁺ alone. Therefore, other monovalent cations such as Na⁺ must be present in the

vacuole to provide the necessary counter-ions.

The accumulation of malic acid in the vacuole is an energy-requiring process (Lüttge & Ball, 1979; Lüttge *et al.*, 1982), whereas efflux of malic acid out of the vacuole is passive (Lüttge & Ball, 1977). It is thought that light acts as the trigger for malic acid release and decarboxylation, although it has been shown that the turgor created by malic acid accumulation may also play a role (Kluge & Ting, 1978).

1.1.1.2. The processes of the light period

(a) *Deacidification and malate decarboxylation.* After the nocturnal accumulation of large quantities of malic acid in the vacuole, there is a rapid and marked decrease in stored malic acid when the plants are exposed to light the following day. The increase in the cytosolic concentration of malic acid prevents further synthesis of malate by feedback inhibition of PEP carboxylase. Deacidification of the vacuole occurs because of malate decarboxylation and the concomitant release of CO₂. In most CAM plants the principal enzyme involved in the decarboxylation of malic acid is either NADP⁺- or NAD⁺-dependent malic enzyme (EC 1.1.1.39 and EC 1.1.1.40, respectively). However, in the case of the Liliaceae, Bromeliaceae, Asclepiadaceae and some other CAM plants, PEP carboxykinase (EC 4.1.1.49) serves as the major decarboxylase. This enzyme has not been detected in CAM plants with high NADP⁺- or NAD⁺-dependent malic enzyme activity and it is possible to classify CAM plants accordingly.

(b) *The fate of the decarboxylation products.* The CO₂ released by the decarboxylation of malic acid is rapidly refixed by Rubisco and incorporated into the Calvin cycle, resulting in the resynthesis of soluble carbohydrates

and glucose.

The pyruvate produced as a by-product of malate decarboxylation is converted directly to carbohydrate by a reversal of glycolysis. The conversion of pyruvate to PEP is catalysed by the enzyme pyruvate orthophosphate dikinase (EC 2.7.9.1), bypassing the irreversible pyruvate kinase of normal glycolysis (see Fig. 1.1). This enzyme has only been detected in CAM plants with high NADP⁺- or NAD⁺- malic enzyme activity. In CAM plants with high PEP carboxykinase activity, the products of malic acid decarboxylation are CO₂ and PEP. Thus there is no requirement for pyruvate orthophosphate dikinase, and this enzyme has not been detected in these plants.

1.1.2. Malic acid transport at the tonoplast

1.1.2.1. Thermodynamics and energetics

The transport of malic acid into the vacuoles of CAM cells is dependent upon the proton electrochemical gradient at the tonoplast,

$$\Delta\mu_{H^+} = \Delta\Psi - \frac{2.3 RT}{F} \Delta pH$$

where F is the Faraday constant, R is the gas constant and T is the absolute temperature. ΔpH is the pH gradient across the vacuole and $\Delta\Psi$ is the membrane potential at the tonoplast, equal to about +25 mV, as determined by measuring transcellular electrical profiles in *Kalanchoë daigremontiana* (Rona *et al.*, 1980). Vacuolar pH can be readily obtained from measurements on leaf sap, since the cytoplasmic volume is very small, i.e. only 0.5 to 1.0% of total cell volume in *K. daigremontiana* (Steudle *et al.*, 1980). Of

course, vacuolar pH varies much during the day-night rhythm of CAM. In *K. daigremontiana* extreme values observed are pH 6.0, at the end of the light period, and pH 3.5, at the end of the dark period (Lüttge *et al.*, 1981).

Cytoplasmic pH can be assumed to be about 7.5. This allows calculation of $\Delta\mu_{H^+}$ at the tonoplast with sufficient accuracy.

To assess the energetics of nocturnal malic acid accumulation as driven by an H^+ -pumping ATPase at the tonoplast, $\Delta\mu_{H^+}$ must be related to ΔG_{ATP} .

ΔG_{ATP} is the free energy available from ATP hydrolysis in the cytoplasm, which is the sum of the standard Gibbs free energy change of ATP hydrolysis, ΔG°_{ATP} , and the mass action ratio for ATP hydrolysis ($[ADP] [P_i]/[ATP]$) as follows:

$$\Delta G_{ATP} = \Delta G^{\circ}_{ATP} + 2.3 RT \log \left(\frac{[ADP] [P_i]}{[ATP]} \right)$$

In *Kalanchoë* species, ΔG_{ATP} was estimated to be about -54 kJ/ mol (Lüttge, 1987). The ratio $-\Delta G_{ATP}/ \Delta\mu_{H^+}$ then gives the force ratio at static head for ATP hydrolysis (Baccarini-Melandri *et al.*, 1977; Rottenberg, 1979). This ratio indicates the thermodynamic limits for the number of protons that can be transported per ATP hydrolysed.

Simultaneous measurements of the malate content and pH of vacuolar sap can be made at regular intervals during the day-night cycle. This allows $\Delta\mu_{H^+}$ to be calculated and $-\Delta G_{ATP}/ \Delta\mu_{H^+}$ to be related to the degree of malate accumulation attained at any time of the rhythm. In *K. daigremontiana* the $-\Delta G_{ATP}/ \Delta\mu_{H^+}$ ratios suggested that for most of the malate accumulation period only a pumping of 1 or 2 H^+ / ATP was possible (Lüttge, 1987). For *K. daigremontiana* and *Kalanchoë tubiflora*, plots of malate levels vs $\Delta\mu_{H^+}$

produce curves which, at higher malate levels, asymptotically approach a $\Delta\mu_{H^+}$ of ~ 54 kJ/ mol but never pass it (Lüttge *et al.*, 1981; Smith *et al.*, 1982; Lüttge & Ball, 1987). This is consistent with a proton-pumping stoichiometry of 2 H^+ / ATP, since beyond 54 kJ/ mol only a transport of 1 H^+ / ATP would remain feasible. A stoichiometry of 2 H^+ / ATP was confirmed for the tonoplast ATPase of *K. tubiflora* by direct measurements of the trans-tonoplast electrical potentials ($\Delta\Psi$) and intra-vacuolar pH of isolated vacuoles (Jochem *et al.*, 1984).

In summary, consideration of the thermodynamic factors shows that nocturnal malate uptake into the vacuoles of CAM plants can be driven by an ATP-dependent H^+ -pump with a stoichiometry of 2 H^+ / ATP (Lüttge, 1987).

1.1.2.2. Components of tonoplast membranes

Tonoplast membranes have been purified from the CAM species *K. daigremontiana* and *Mesembryanthemum crystallinum* by isolation of whole intact vacuoles from isolated protoplasts (Aoki & Nishida, 1984; Smith *et al.*, 1984a,b) and by preparation of tonoplast homogenates on continuous and discontinuous sucrose gradients (Jochem & Lüttge, 1987; Marquardt & Lüttge, 1987; Struve & Lüttge, 1987). Investigation of these purified tonoplast vesicles has led to the detection and biochemical characterisation of two novel eukaryotic proton pumps in this membrane : a H^+ -translocating inorganic pyrophosphatase (PP_i -ase) and a H^+ -translocating ATPase. Malate transport has also been studied in these tonoplast vesicles and a malate carrier has been found in tonoplast vesicles isolated from *K. daigremontiana* (Buser-Suter *et al.*, 1982).

1.1.2.3. The tonoplast PP_i-ase

An electrogenic proton-pumping PP_i-ase has been found in the tonoplast vesicles of *K. daigremontiana* (Marquardt & Lüttge, 1987) and has been characterised in detail. The substrate is MgPP_i²⁻ and the pH optimum is slightly alkaline (~ pH 8.0). The PP_i-ase has an absolute requirement for high levels of K⁺ (≥ 50 mM) and is insensitive to anions (Marquardt & Lüttge, 1987; White *et al.*, 1990).

1.1.2.4. The tonoplast ATPase

ATP hydrolytic activity has been detected in tonoplasts from both the CAM species mentioned above. The substrate for the ATPase is MgATP²⁻ and the pH optimum is slightly alkaline (~ pH 8.0). There is no cation requirement other than the divalent cation in the substrate. Monovalent anions are highly effective, NO₃⁻ being inhibitory and Cl⁻ and malate stimulatory (Aoki & Nishida, 1984; Smith *et al.*, 1984*a,b*; Jochem *et al.*, 1984; Struve *et al.*, 1985; Jochem & Lüttge, 1987; Struve & Lüttge, 1987; White & Smith, 1989).

ATP-dependent proton-pumping in tonoplast vesicles has been demonstrated using fluorescent dyes such as 9-aminoacridine and quinacrine (Wang & Sze, 1985; Jochem & Lüttge, 1987; White & Smith, 1989). These fluorescent amines are weak bases, which are lipid-permeant in their neutral form but become impermeant in their protonated form. Thus, in the absence of a pH gradient the concentrations of the protonated and unprotonated form of the probe inside a vesicle are equal to those in the outside medium at equilibrium. If a pH gradient (acid inside) is generated, the concentration of the protonated species increases within the vesicles, since it is impermeant (Fig. 1.2). Accumulation of the amine results in

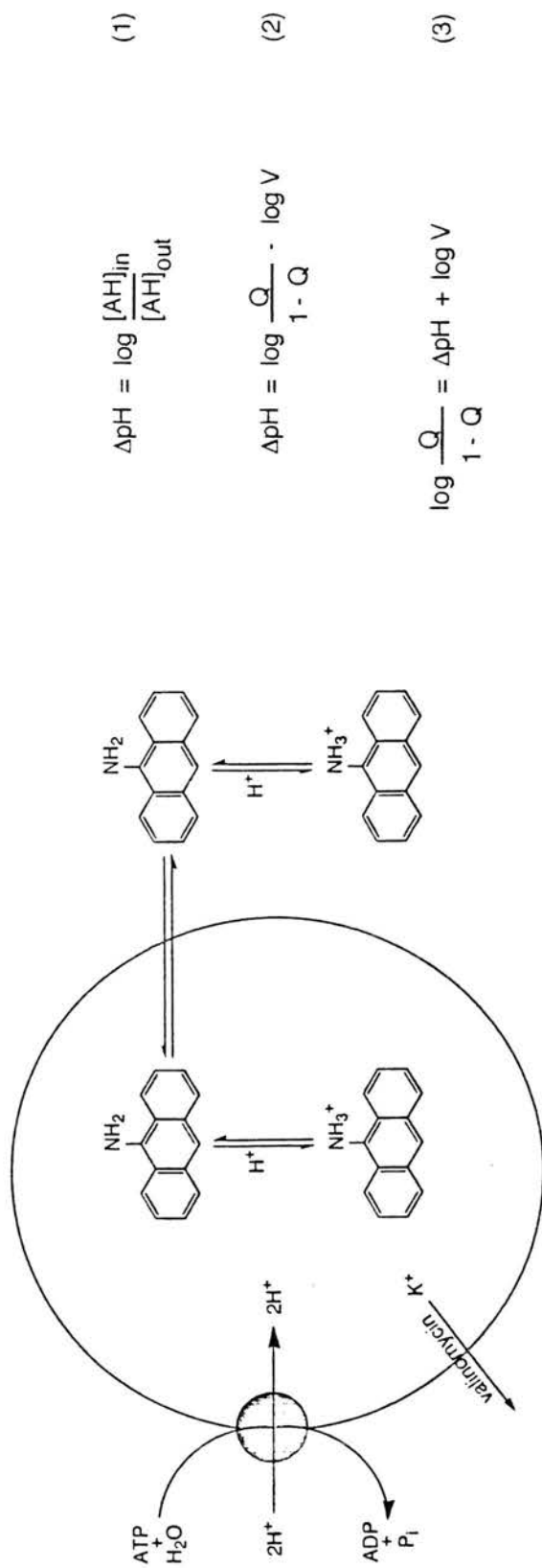


Fig. 1.2. Fluorescence quenching of 9-aminoacridine in response to a pH gradient generated by a H⁺-translocating ATPase.

Left, a schematic diagram of the partitioning of 9-aminoacridine into membrane vesicles. Right, a derivation of the Schuldiner equation, which gives a quantitative description of the quenching of fluorescence in response to ΔpH . $[AH]_{in}$ and $[AH]_{out}$ are the concentrations of the protonated amine inside and outside of the vesicles, respectively. Q is the fraction of the total fluorescence that is quenched and V is the fractional volume of the osmotic compartment, relative to the total volume of the solution. The substitution of Q and V for $[AH]_{in}$ and $[AH]_{out}$ assumes that the internalised amine is not fluorescent.

quenching of the fluorescence intensity through molecular stacking: an overall fall in the fluorescence of the solution is recorded. The extent of quenching can be related to the ΔpH across the membrane according to the expression (Schuldiner *et al.*, 1972):

$$\log (Q/(1-Q)) = \text{pH}_{\text{out}} - \text{pH}_{\text{in}} + \log V$$

where Q is the fraction of the total fluorescence that is quenched and V is the fractional volume of the osmotic compartment, relative to the total volume of the solution.

The dye oxonol VI has been used to confirm the generation of an ATP-dependent inside-positive tonoplast membrane potential. The oxonol dyes are a class of permeant anions which have been shown to respond rapidly and quantitatively to positive interior membrane potentials (Bashford *et al.*, 1979; Scherman & Henry, 1980), of which oxonol VI has been shown to be the most sensitive of these dyes (Bashford & Thayer, 1977). A change in the positive interior membrane potential ($\Delta\Psi$) is measured as a shift in the absorbance spectrum (measured as absorbance difference at 610 and 580 nm) of oxonol VI and quenching of its fluorescence. The response of oxonol dyes to changes in $\Delta\Psi$ apparently depends on shifts between the free and membrane-bound dye. At neutral pH oxonol dyes are anions which distribute across the vesicle membrane in accordance with $\Delta\Psi$. An increase in $\Delta\Psi$ leads to the accumulation of dye in the vesicle and shifts the dye-membrane equilibrium in favour of increased dye binding and this is probably the cause of the spectral shift.

The use of these probes in studying the tonoplast ATPase has shown that it is an electrogenic proton pump. Both the tonoplast ATPase and PP_i -ase are capable of generating a transmembrane H^+ -electrochemical potential

difference ($\Delta\mu_{H^+}$) which could be utilised to drive the nocturnal uptake of malic acid into the vacuole. The tonoplast ATPase is of particular interest since it is a member of the “vacuolar” class (V-type) of H^+ -ATPases, which are associated with endomembrane systems of higher eukaryotes (see section 1.2.3).

1.1.2.5. Malate transport

The $MgATP^{2-}$ -dependent uptake of malate into isolated plant vacuoles has been demonstrated for the mesophyll of barley (Martinoia *et al.*, 1985) and the CAM plant *K. daigremontiana* (Nishida & Tominga, 1987).

In sealed tonoplast vesicles, malate transport can be shown indirectly by its interaction with $\Delta\mu_{H^+}$. This involves consideration of both the direct and indirect effects of anions upon the $\Delta\mu_{H^+}$ generating ATPase. The direct stimulatory effects of chloride and malate, and the inhibitory effects of nitrate on the tonoplast ATPases of CAM species and other plants have been demonstrated with leaky unsealed vesicles, where $\Delta\mu_{H^+}$ was lowered by the use of a detergent such as Triton or a protonophore such as gramicidin (Bennett & Spanswick, 1983; Aoki & Nishida, 1984; Smith *et al.*, 1984*a,b*; Jochem *et al.*, 1984; Blumwald & Poole, 1985; Lew & Spanswick, 1985; Struve *et al.*, 1985; Jochem & Lüttge, 1987; Struve & Lüttge, 1987; White & Smith, 1989). In sealed vesicles, anions such as chloride and malate, as well as directly stimulating the ATPase complex, can also stimulate the ATPase indirectly by relieving the feedback inhibition of the $\Delta\mu_{H^+}$ generated by the ATPase (Fig. 1.3). Obviously this charge compensation mechanism requires the H^+ -dependent uptake of anions into the sealed vesicles.

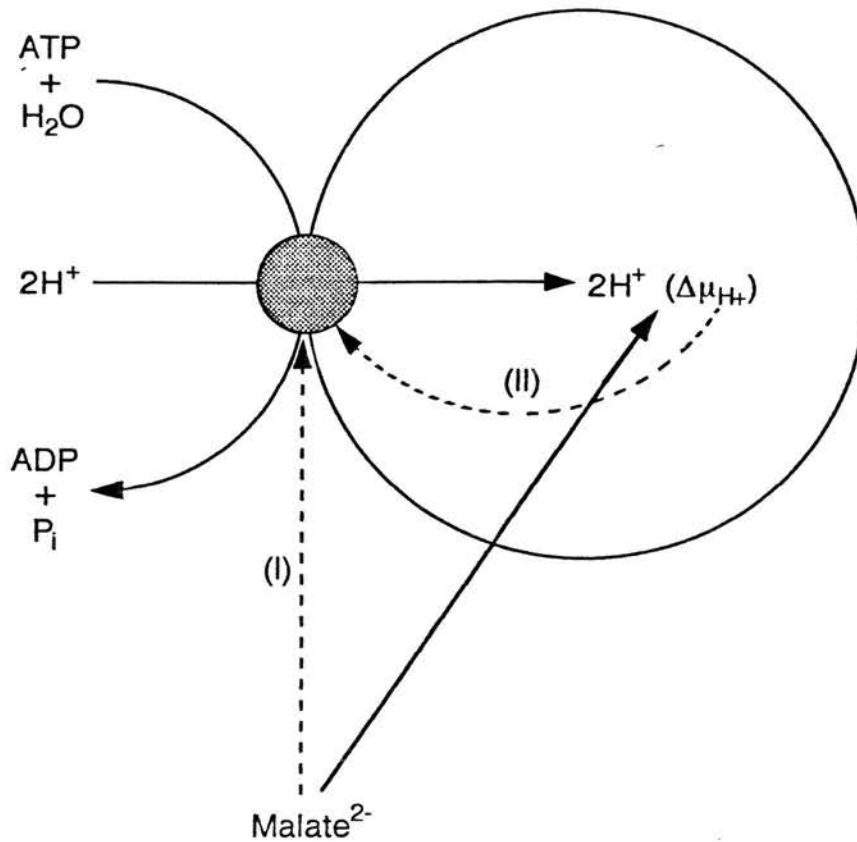


Fig. 1.3. Malate stimulation of the H^+ -transporting ATPase in the membrane of sealed tonoplast vesicles. Malate can stimulate the proton-pump in two ways; firstly, by the direct action of malate anions on the ATPase complex (I); and secondly, by alleviating the inhibitory effect of $\Delta\mu_{\text{H}^+}$ (II), as $\Delta\mu_{\text{H}^+}$ is used for malate uptake.

Since there are both direct and indirect anion effects on the tonoplast ATPase, ATP hydrolysis should be stimulated in the presence of a detergent, but there should be additional stimulation in its absence due to the dissipation of $\Delta\mu_{H^+}$ by anion uptake. This has been shown for chloride and malate, using tonoplast preparations of *M. crystallinum* with Triton as the detergent (Struve & Lüttge, 1987). Malate has also been shown to release the inside-positive electrical potential in tonoplast vesicles of *K. daigremontiana* (Jochem & Lüttge, 1987).

MgPP_i²⁻-dependent uptake of malate in sealed vesicles is simpler to explain since the PP_i-ase is not affected by anions. This stimulation of H⁺-transport by malate clearly indicates that malate uptake is driven by components of $\Delta\mu_{H^+}$ (Marquardt & Lüttge, 1987).

Overall consideration of these results clearly supports the occurrence of a $\Delta\mu_{H^+}$ -driven malate transport mechanism at the tonoplast of CAM cells. The efflux of malate from the vacuole during the following daylight period is a passive process (Lüttge & Ball, 1977; Lüttge & Ball, 1979), although the exact mechanism by which this occurs is not fully understood (Lüttge & Smith, 1984).

1.2. The role of ATPases in the cell

Virtually all biological systems utilise ATP either directly or indirectly as an energy source. Since most cell types are impermeable to ATP, it must be recycled within each cell. Thus all known cell types perform an ATP synthesis/ ATP hydrolysis cycle of the type shown in Fig. 1.4. This cycle is essential for maintaining the viability of the cell and, therefore to the function of intact prokaryotic and eukaryotic organisms.

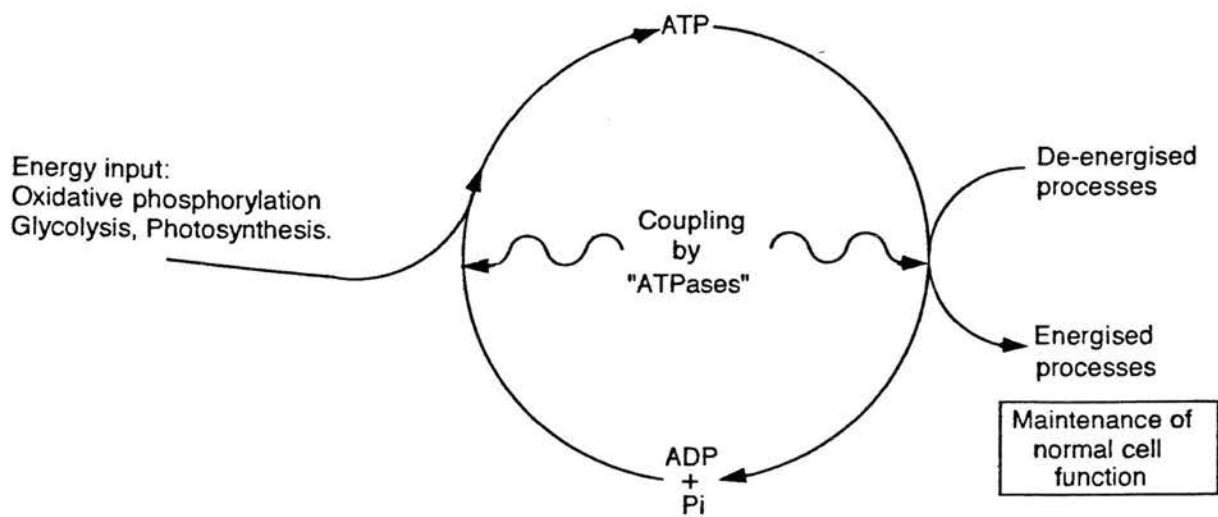


Fig. 1.4. ATP synthesis/ ATP hydrolysis cycle catalysed by most living cells.

The ATP synthesis/ ATP hydrolysis cycle of each cell must be able to respond immediately to the energy demands of the organism at any given time, and to operate in a tightly coupled manner. This is achieved by a wide variety of ATPases of which there are three major classes, designated, P, V and F, within the cell. These three types of ATPases are coupled by ATP in a master-slave relationship (Fig. 1.5). The F-type ATPase located in the inner mitochondrial membrane (and the thylakoid membrane of chloroplasts) works solely as an ATP synthase and acts as the 'master', supplying ATP to the extra- mitochondrial 'slave' ATPases. These comprise the P-type ATPases found in the plasma membrane and the endoplasmic and sarcoplasmic reticula, and the V-type ATPases which are associated with intracellular organelles other than mitochondria, E.R. and S.R. The P-type and V-type ATPases function solely as ATP hydrolases, energising the processes necessary for the maintenance of normal cell function (Pedersen & Carafoli, 1987).

1.2.1. P-type ATPases

P-type ATPases are defined as those which form a phosphorylated intermediate as part of their reaction cycle. This class includes a variety of cation-specific pumps, such as the Na^+/K^+ -, Ca^{2+} - and H^+ -translocating ATPases of the plasma membranes of eukaryotic cells, Ca^{2+} -ATPase of the sarcoplasmic recticulum, H^+/K^+ -ATPase of the gastric mucosa and the K^+ -ATPases of *Escherichia coli* and *Streptococcus faecalis* (Pedersen & Carafoli, 1987; Nelson & Taiz, 1989; Forgac, 1989). Structurally, all of these ATPases consist of a catalytic subunit of molecular weight 90-140 kDa. In most cases this polypeptide has a molecular weight of approximately 100 kDa and is the only subunit required for ATP-driven ion transport.

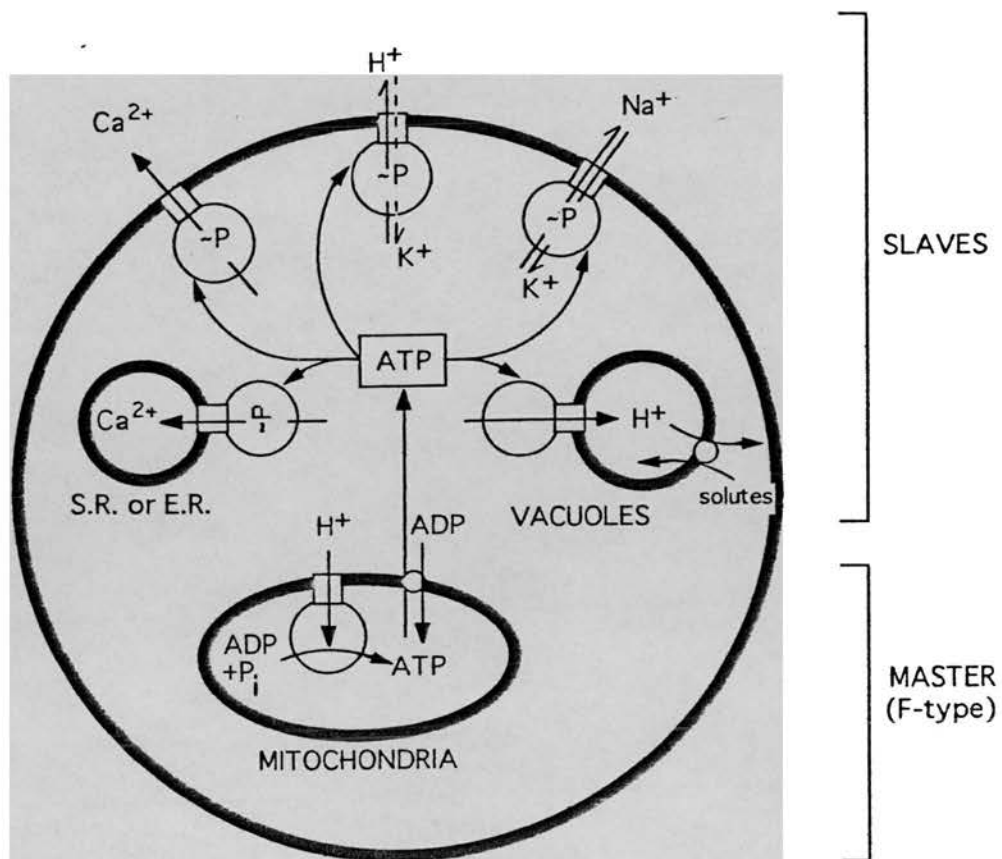


Fig. 1.5. Coupling relationship between the "master" H^+ -ATPase (F-type) and the "slave" ATPases.

There are three exceptions, the plasma membrane Ca^{2+} -ATPase which has a molecular weight of 140 kDa; the $\text{Na}^{+}/\text{K}^{+}$ -ATPase which has an additional glycosylated subunit of 50 kDa; and the *E. coli* K^{+} -ATPase which requires two additional subunits for ion transport (Forgac, 1989).

During the reaction cycle of P-type ATPases a phosphorylated enzyme intermediate is formed. The catalytic subunit is phosphorylated by the terminal phosphate of ATP being transiently attached to the carboxyl group of an aspartyl residue present at the active site. This phosphorylation reaction is stimulated by the presence on the cytoplasmic face of the membrane of the ion to be transported from the cytoplasmic to the extracytoplasmic surface. For example, phosphorylation of the $\text{Na}^{+}/\text{K}^{+}$ - and Ca^{2+} -ATPases is stimulated by the binding to high-affinity sites of cytoplasmic Na^{+} and Ca^{2+} , -respectively (Forgac, 1989).

P-type ATPases are characteristically inhibited by vanadate at concentrations $\leq 100 \mu\text{M}$ (Forgac, 1989). Vanadate is thought to act as a transition state analogue of phosphate for these enzymes (Pedersen & Carafoli, 1987; Stone & Xie, 1988; Forgac, 1989).

1.2.2. F-type ATPases

F-type ATPases (F_1F_0 -type) are found in the plasma membranes of bacteria and the inner membranes of mitochondria and chloroplasts. They function as ATP synthases, harnessing the transmembrane electrochemical proton gradient across the membrane to drive the synthesis of ATP (Pedersen & Carafoli, 1987; Nelson & Taiz, 1989; Forgac, 1989). They can also operate in the reverse direction, hydrolysing ATP and pumping protons across the membrane. This may be important for bacteria growing

fermentatively, but is unlikely to be so for mitochondria and chloroplasts.

These enzymes operate without forming a phosphorylated intermediate (Pedersen & Carafoli, 1987; Stone & Xie, 1988). F-type ATPases are structurally more complex than P-type ATPases. They are multisubunit complexes of ~ 500 kDa, containing at least eight different subunits (Pedersen & Carafoli, 1987; Forgac, 1989). These proton pumps can be divided into two functionally distinct domains. The hydrophilic catalytic complex (F_1) which in *E. coli* contains five subunits of molecular weights 55 kDa (α), 50 kDa (β), 31 kDa (γ), 20 kDa (δ) and 15 kDa (ϵ) in the stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$; and the integral membrane proton conduction channel (F_0) which in the *E. coli* enzyme consists of three subunits of molecular weights 30 kDa (a), 17 kDa (b) and 8 kDa (c) in the stoichiometry ab_2c_{6-12} (Nelson & Taiz, 1989; Forgac, 1989).

Both the α and β subunits of the F_1 complex have been shown, by direct binding studies and the use of nucleotide-analogue inhibitors, to contain nucleotide-binding sites. The β subunit appears to contain the catalytic site, while the nucleotide site on the α subunit may have some regulatory function (Nelson & Taiz, 1989; Forgac, 1989). The γ , δ and ϵ subunits have yet to be ascribed any specific function, but are thought to form at least part of the "stalk" region which connects the F_1 and F_0 domains. Therefore they are believed to be involved in coupling the energy released from ATP hydrolysis in F_1 to the movement of protons through F_0 (Forgac, 1989).

The F_0 domain of F-type ATPases has been studied extensively. The c-subunit is believed to be directly involved in proton-translocation (Forgac, 1989). It has been shown that the covalent modification by *N,N'*-dicyclohexylcarbodiimide (DCCD) of a single glutamic or aspartic acid carboxyl

group on only one of the c-subunits is sufficient to block proton translocation through the entire F_0 complex (Forgac, 1989; see Fig. 1.6). This suggests that it requires the cooperative interaction of multiple copies of the c-subunit to form a functional proton pore. However, evidence from both genetic and reconstitution data show that the a and b subunits are also required for the formation of a functional proton channel (Pedersen & Carafoli, 1987; Forgac, 1989).

Apart from DCCD, F-type ATPases are also inhibited by aurovertin, efrapeptin, oligomycin, venturicidin, trialkyltin, diethylstilbestrol (DES) and various mercurial agents (Pedersen & Carafoli, 1987). Aurovertin and efrapeptin inhibit the F_1 domain, while the other inhibitors act on the F_0 domain of the ATPase complex. Oligomycin, venturicidin and trialkyltin all bind to the c-subunit (DCCD reactive protein). The sites of action of DES and the mercurials is unknown. The sensitivity of bacterial and eukaryotic F-type ATPases to these subunits differs in some cases. For example, oligomycin, a potent inhibitor of all animal F-type ATPases, has little effect on the *E. coli* enzyme.

1.2.3. V-type ATPases

V-type ATPases are a novel class of proton pump present in the endomembrane systems of eukaryotic cells (Forgac 1989). In animal cells these proton pumps are responsible for acidifying a variety of intracellular compartments including clathrin-coated vesicles (Forgac & Cantley, 1984; Stone *et al.*, 1986), Golgi derived vesicles (Glickman *et al.*, 1983) and chromaffin granules (Cidon & Nelson, 1983; Percy *et al.*, 1985). They are also present in the vacuolar membranes of plants (Aoki & Nishida, 1984; Smith *et al.*, 1984a,b; Mandala & Taiz, 1985b; Manolson *et al.*, 1985; Marin

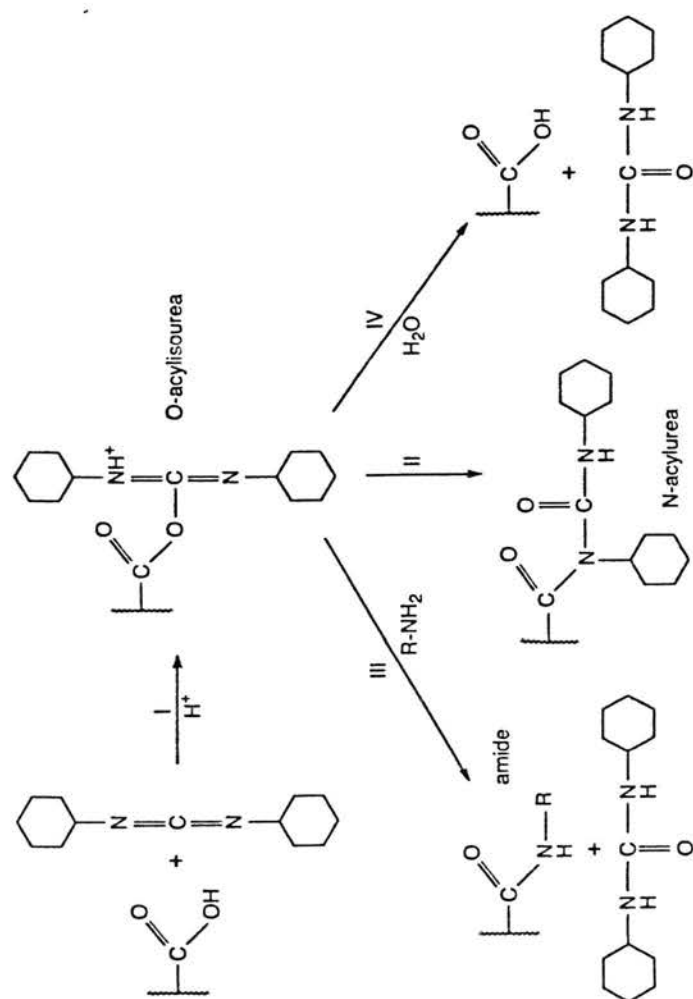


Fig. 1.6. Interaction of DCCD with a carboxyl residue. When DCCD reacts with a carboxyl residue, an unstable O-acylisourea adduct is first formed (I), which can then rearrange to a stable N-acylurea (II). Only this reaction sequence results in the incorporation of radioactivity when radiolabelled DCCD is employed. The O-acylisourea adduct can also interact with a nucleophile, such as a nearby ϵ -amino group of a lysine residue, to form an amide and dicyclohexylurea (III). If the nucleophile is water, the free carboxyl residue is restored (IV).

et al., 1985; Wang & Sze, 1985) and fungi such as *Neurospora* (Bowman, 1983) and Yeast (Uchida *et al.*, 1985, Lichko & Okorokov, 1985).

Exceptionally, V-type ATPases are found in the plasma membrane (Stone & Xie, 1988; Wieczorek *et al.*, 1989; Väänänen *et al.*, 1991). The properties of these enzymes distinguish them from both the P- and F-type ATPases, although structural information suggests that V-type and F-type ATPases may have a common evolutionary ancestor (Gogarten *et al.*, 1989).

Intracellular organelles utilise the transmembrane pH-difference in accordance with their function. Thus exposure to low pH within endosomes causes dissociation of ligand/receptor complexes after receptor-mediated endocytosis, facilitating receptor recycling. Acid-activated dissociation is also involved in the recycling of receptors for lysosomal enzymes to the Golgi after delivery of their ligands to vesicles targeted for the lysosome. Exposure to low pH within the endocytotic compartment is a critical signal in activating the entry of various pathogens such as certain viruses and toxins into the cytoplasm of the host cell. In secretory vesicles, low pH is important in the uptake of certain small molecules, and in the proteolytic processing of certain peptides. Lysosomes and the vacuoles of plants and fungi contain a variety of hydrolytic enzymes which require an acidic pH for optimal activity. In addition, the negative (i.e. inside-positive) membrane potential generated by the acidification of the vacuolar compartments of plants and lower eukaryotes is believed to be important in the accumulation of small molecules stored in the vacuoles.

1.2.3.1. Inhibitor specificity of V-type ATPases

Vacuolar H⁺-ATPases can be distinguished from those of the P-type and F-type by their inhibitor sensitivity. Thus all V-type ATPases are resistant to

vanadate (Walker & Leigh, 1981; Gluck *et al.*, 1982; Bowman, 1983; Forgac *et al.*, 1983; Galloway *et al.*, 1983; Glickman *et al.*, 1983; Yamashiro *et al.*, 1983; Smith *et al.*, 1984a,b; Mandala & Taiz, 1985b; Percy *et al.*, 1985; Uchida *et al.*, 1985; Wang & Sze, 1985; Schweikl *et al.*, 1989), the characteristic inhibitor of P-type ATPases, which is believed to function as a transition-state analogue. The resistance of V-type ATPases to vanadate suggests that these enzymes, like F-type ATPases, do not undergo active-site phosphorylation.

V-type ATPases are also resistant to several characteristic inhibitors of F-type ATPases, including oligomycin (Walker & Leigh, 1981; Gluck *et al.*, 1982; Forgac *et al.*, 1983; Glickman *et al.*, 1983; Smith *et al.*, 1984a; Mandala & Taiz, 1985a; Uchida *et al.*, 1985) and azide (Walker & Leigh, 1981; Bowman, 1983; Smith *et al.*, 1984b; Uchida *et al.*, 1985; Wang & Sze, 1985; Xie & Stone, 1986; Wieczorek *et al.*, 1989).

V-type ATPases are particularly sensitive to alkylating agents such as 7-chloro-4-nitrobenzofurazan (Nbf-Cl) (Glickman *et al.*, 1983; Bowman *et al.*, 1985; Uchida *et al.*, 1985; Mandala & Taiz, 1986; Arai *et al.*, 1987; Randall & Sze, 1987; Cidon & Sihra, 1989; Padh *et al.*, 1989; Simon & Burckhardt, 1990) and *N*-ethylmaleimide (NEM) (Galloway *et al.*, 1983; Glickman *et al.*, 1983; Bowman *et al.*, 1985; Griffith *et al.*, 1985; Xie & Stone, 1986; Arai *et al.*, 1987; Moriyama & Nelson, 1987; Cidon & Sihra, 1989; Padh *et al.*, 1989; Wieczorek *et al.*, 1989). Both Nbf-Cl and NEM inhibit V-type ATPases in an ATP- protectable fashion. This has enabled the catalytic subunit of these enzymes to be tentatively identified, although it is possible to explain these results in other ways; for example, the NEM-sensitive ATP-binding site might be regulatory.

V-type ATPases are characteristically inhibited by nitrate (Bowman, 1983; Smith *et al.*, 1984a; Bennett *et al.*, 1985; Lew & Spanswick, 1985; Mandala

& Taiz, 1985b; Wang & Sze, 1985; Griffith *et al.*, 1986; Moriyama & Nelson, 1987; Struve & Lüttge, 1987; Arai *et al.*, 1989; Cidon & Sihra, 1989; Kane *et al.*, 1989) and the macrolide antibiotic bafilomycin A₁ (Bowman *et al.*, 1988c; Kane *et al.*, 1989; Moriyama & Nelson, 1989c; Pérez-Castiñeira & Apps, 1990; Warren *et al.*, 1992; Ward & Sze, 1992). Typically, 50 % inhibition of V-type ATPases is achieved at nitrate concentrations of 1-100 mM. Bafilomycin inhibits ATPase activity at concentrations of 0.1-1 ng bafilomycin/μg protein, equivalent to about 1 mol/mol, though its mechanism of action is unknown.

DCCD is also a potent inhibitor of V-type ATPases (Sutton & Apps, 1981; Bowman, 1983; Smith *et al.*, 1984a; Uchida *et al.*, 1985; Mandala & Taiz, 1986; Arai *et al.*, 1987; Rea *et al.*, 1987; Kaestner *et al.*, 1988; Wieczorek *et al.*, 1989). As with F-type ATPases, DCCD appears to inhibit the V-type ATPase by blocking proton translocation through covalent modification of the smallest subunit (see Fig. 1.6).

1.2.3.2. Ion transport specificity

The V-type ATPases are electrogenic proton pumps. This has been demonstrated by the dependence of proton transport and ATPase activity upon the addition of the ionophore valinomycin (in the presence of K⁺), which dissipates the membrane potential established during ATP-driven proton transport (D'Souza *et al.*, 1982; Lichko & Okorokov, 1984; Xie *et al.*, 1986; Young *et al.*, 1988; Pérez-Castiñeira & Apps, 1990) and thereby stimulates H⁺-translocation. In the case of intact vacuolar organelles acidification shows a requirement for the presence of a permeant anion, typically chloride (Walker & Leigh, 1981; Churchill *et al.*, 1983; Jochem *et al.*,

1984; Moriyama & Nelson, 1987; Arai *et al.*, 1989). This anion requirement could be due either to a direct Cl^- stimulation of H^+ -ATPase activity or to the dissipation of the vacuolar inside-positive membrane potential established during ATP-driven proton transport, by means of passive Cl^- entry, or entry through an anion channel. Experiments using purified reconstituted H^+ -ATPase from clathrin coated vesicles have shown that this enzyme is capable of ATP-dependent proton translocation in the complete absence of Cl^- provided a charge compensation mechanism is present to dissipate the membrane potential generated by proton translocation. This can be provided by internal K^+ and valinomycin. Even at high concentrations of Cl^- proton translocation only occurred in the presence of K^+ and valinomycin (Arai *et al.*, 1989). These results indicate that the anion channel which provides the charge compensatory Cl^- flux in the vesicles is not a component of the purified H^+ -ATPase; furthermore, the dependence of H^+ -translocation on anion flux has been used as an assay in the purification of the chloride channel (Xie *et al.*, 1989).

1.2.3.3. Determination of the H^+ /ATP stoichiometry for V-type ATPases

Consideration of the thermodynamics of nocturnal malate uptake into the vacuole of CAM plants has led to the determination of a 2H^+ /ATP stoichiometry for the tonoplast H^+ -ATPase of *Kalanchoë* species (see section 1.1.3.1).

The H^+ /ATP stoichiometry of the H^+ -ATPase from chromaffin granules has also been extensively studied. Flatmark and Ingebretson (1977)

measured an H^+/ATP ratio of 1.58 in chromaffin granule “ghosts” after a long incubation, in the presence of a high concentration of ATP and utilising the distribution of the membrane permeable dye bromothymol blue from which the proton flux was back-calculated. Njus *et al.*, (1978) calculated an H^+/ATP ratio of 1 in intact granules which already possessed an endogenous pH gradient using large ATP concentrations, long incubation times and regression analysis to calculate the back leak. Finally, Johnson *et al.*, (1982) measured the H^+/ATP stoichiometry in resealed chromaffin granule ghosts devoid of ATP and catecholamines under conditions where no net pH changes occur upon ATP hydrolysis. Their results revealed a H^+/ATP ratio as high as 1.8, indicating that the H^+/ATP ratio is most likely 2.0.

The coupled reactions of ATP hydrolysis and proton-translocation are related by the expression:

$$\Delta G_{ATP} = -nF\Delta\mu_{H^+}$$

where ΔG_{ATP} is the free energy of ATP hydrolysis, n is the H^+/ATP ratio and $\Delta\mu_{H^+}$ is the proton electrochemical gradient. Therefore, for proton-translocation a low H^+/ATP ratio will allow a given ΔG_{ATP} to pump protons against a large $\Delta\mu_{H^+}$ (Cross & Taiz, 1990). Thus a stoichiometry of 2 is in good agreement with these thermodynamic requirements.

1.2.3.4. Structure of V-type ATPases

The vacuolar H^+ -ATPases from a wide variety of sources have now been purified. These enzymes are of high molecular weight (400-600 kDa) and

complex subunit composition (see Table 1.1). All V-type ATPases contain at least three subunit types i) 66-73 kDa; ii) 55-62 kDa; and iii) 13-17 kDa. Subunits of intermediate size (20-40 kDa) are also present in most preparations. In some cases up to five polypeptides in this range have been reported. Controversy surrounds the largest (100-120 kDa) subunit, which originally was found only in the V-type ATPases from chromaffin granules (Cidon & Nelson, 1983; Apps *et al.*, 1989) and clathrin-coated vesicles (Arai *et al.*, 1988). However, this subunit has since been detected in the V-type ATPase from kidney microsomes (Gillespie *et al.*, 1991), and a polypeptide of comparable size has been reported to occur in V-type ATPases from red beet (Parry *et al.*, 1989) and yeast vacuoles (Kane *et al.*, 1989). The presence of a "large" subunit in the ATPase from red beet and yeast is interesting since most groups working with plant or fungal V-type ATPases have not detected a subunit of this size in their preparations.

Topographical studies of the V-type ATPases from chromaffin granules and clathrin-coated vesicles have been carried out using membrane-impermeant reagents and the hydrophobic reagent 3-(trifluoromethyl)-3-(iodophenyl)diazirine (TID) (Arai *et al.*, 1988; Apps *et al.*, 1989). Membrane-impermeant reagents labelled those ATPase subunits exposed on the cytoplasmic surface of intact vesicles. Those polypeptides exposed to the luminal surface were identified by increased labelling by these reagents either in membrane fragments or following solubilisation under non-denaturing conditions. Labelling by TID was used to indicate which subunits were embedded in the lipid bilayer. Similar results were obtained for the clathrin-coated vesicle ATPase and the chromaffin granule ATPase. The 72 kDa and 57 kDa subunits were the principal proteins labelled on the cytoplasmic face of the membrane. The 100-120 kDa subunit also showed cytoplasmic labelling. Treatment with neuraminidase and endoglyco-

Source	M _r (kDa)	Subunits: apparent mol. wt. (kDa)					Reference				
Chromaffin granule	500	115	72	57	41	33	16	(a)			
Clathrin-coated vesicle	530	116	70	58	40	38	34	33	19	17	(b, c)
Kidney microsome			70	56	45	42	38	33	31	15, 14, 12	(d)
Lysosome		115	72	57	41	39	34	33	18, 15	(e)	
<i>Hevea</i> tonoplast	200		66	54					23	13	(f)
<i>Kalanchoë</i> tonoplast			72	57	48	42	39	33	16	(g)	
Maize tonoplast	400		72	62					16	(h)	
Mung bean tonoplast			68	57					16, 13, 12	(i)	
Oat root tonoplast	650		70	60	44, 43	38, 37	32	32	29	16, 13, 12	(j)
Red beet tonoplast		100	67	55	52	44	42	36	32	16	(k)
<i>Neurospora</i> vacuole	520		67	57	51	48			30	16	(l)
<i>S. ceravisiae</i> vacuole		100	69	60		42	36	32	27	17	(m)
Tobacco hornworm midgut			70	57	46				29	17	(n)

Table 1.1. Estimated molecular mass of holoenzyme and subunit composition of isolated vacuolar ATPases. References:

(a) Pérez-Castiñeira & Apps (1990); (b) Arai *et al.* (1987); (c) Stone & Xie (1988); (d) Wang & Gluck (1990); (e) Moriyama & Nelson (1989b); (f) Marin *et al.* (1985); (g) Warren *et al.* (1992); (h) Mandala & Taiz (1986); (i) Matsuura-Endo *et al.* (1990); (j) Ward & Sze (1992); (k) Parry *et al.* (1989); (l) Bowman *et al.* (1989); (m) Kane *et al.* (1989); (n) Schweikl *et al.* (1989).

peptidase F has revealed that this latter subunit is a glycoprotein (Apps *et al.*, 1989; Adachi *et al.*, 1990). More recently it has been shown that the 115 kDa subunit of the kidney microsome V-ATPase is also glycosylated (Gillespie *et al.*, 1991). The glycosylation of the large subunit suggests that it is a transmembrane protein, since oligosaccharide chains are invariably located on the exoplasmic domains of transmembrane proteins. This is supported by the labelling of this subunit with TID. The 17 kDa subunit was labelled on the luminal surface of the membrane and was also strongly labelled by TID suggesting that it forms the bulk of the membrane bound component of the ATPase complex.

These results suggest a model for the V-type ATPase in which the 17 kDa protein is integral to the membrane and the 57 kDa and 72 kDa polypeptides form part of a peripheral complex on the cytoplasmic face of the membrane (see Fig. 1.7).

The structures of V-type ATPases from a wide variety of sources have been studied. By dissociating the ATPase complex, it is possible to determine which subunits are peripheral and which are integral to the membrane. Dissociation of the ATPase complex has usually been achieved by incubation of the enzyme at 4°C in the presence of ATP and/or chaotropic anions such as SCN⁻, I⁻ or NO₃⁻ for 30 to 60 min. This is sometimes referred to as 'cold inactivation'. This treatment has been successfully applied to V-type ATPases from chromaffin granules (Moriyama & Nelson 1989a); clathrin-coated vesicles (Adachi *et al.*, 1990); lysosomes (Moriyama & Nelson 1989b); *Neurospora* (Bowman *et al.*, 1989); oat roots (Lai *et al.*, 1988; Ward & Sze, 1992) and red beet (Rea *et al.*, 1987). The mammalian V-type ATPases all release a set of five polypeptides of apparent molecular weight 72-73 kDa, 57-58 kDa, 40-41 kDa, 34 kDa and 33 kDa. Similarly, six polypeptides of apparent molecular mass 67 kDa, 57 kDa, 51 kDa, 48 kDa,

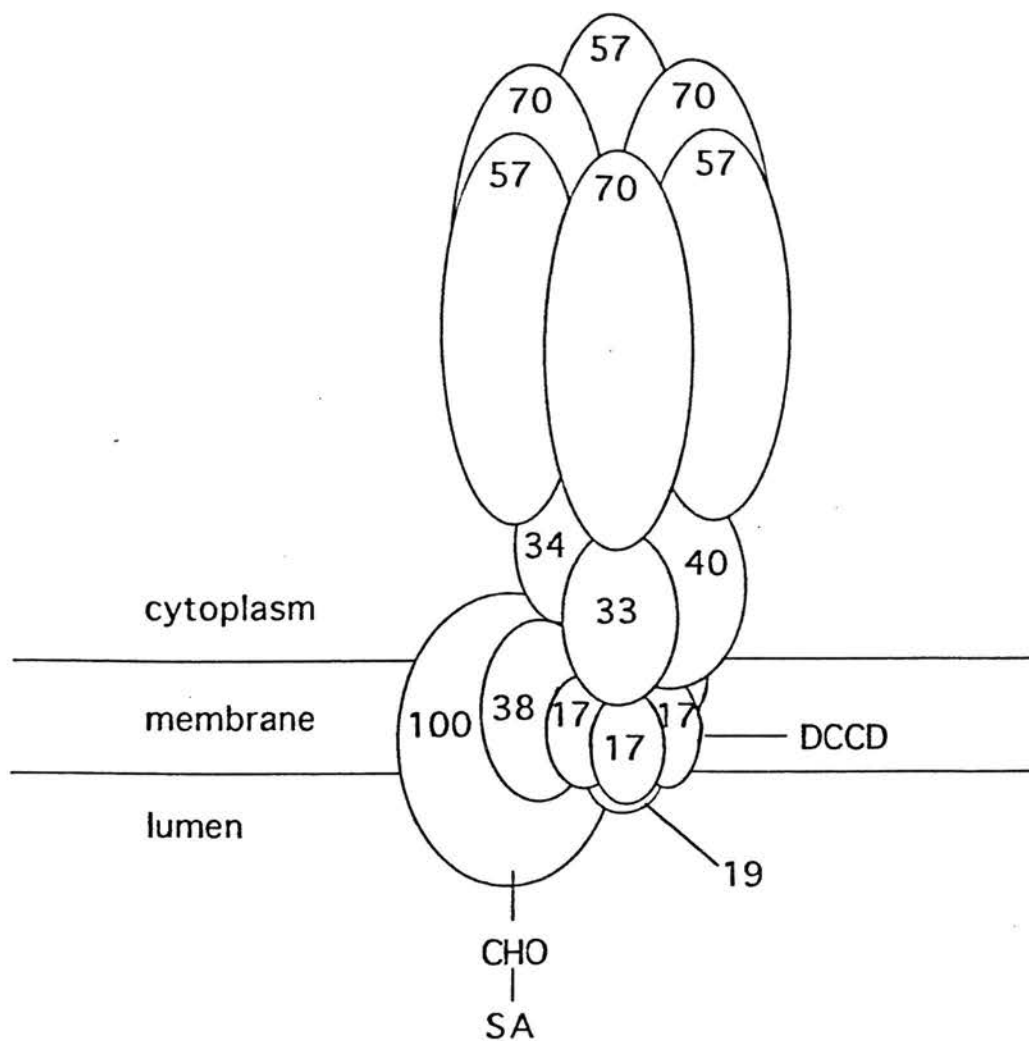


Fig. 1.7. Proposed structural model for vacuolar H^+ -ATPases. The model shown is based largely on the subunit stoichiometry and topographical data obtained for the coated vesicle H^+ -ATPase (Arai *et al.*, 1988; Adachi *et al.*, 1990). The 70, 57, 40, 34 and 33 kDa subunits are all peripheral membrane proteins located on the cytoplasmic side of the membrane. There are three copies each of the 70 and 57 kDa subunits. Both the 70 and 57 kDa subunits possess nucleotide binding sites. The 100, 38, 19 and 17 kDa subunits are all intrinsic membrane proteins. The 17 kDa subunit, of which there are believed to be six copies, is selectively labelled by DCCD and forms at least part of a proton channel. Labelling of both the 100 and 17 kDa subunits by the hydrophobic, photoactivated reagent [125 I] TID indicates that they both possess regions exposed to the lipid bilayer. Moreover, the 100 kDa subunit also possesses covalently bound carbohydrate (CHO) terminating in sialic acid (SA).

30 kDa and 16 kDa are dissociated from the *Neurospora* ATPase (Bowman *et al.*, 1989) and also, six subunits of apparent molecular mass 70 kDa, 60 kDa, 44 kDa, 42 kDa, 36 kDa and 29 kDa are dissociated from the oat root ATPase (Ward & Sze, 1992). However, only two polypeptides of, 67 kDa and 57 kDa are released from the red beet enzyme (Rea *et al.*, 1987).

Interestingly, analysis of the six peripheral polypeptides from the *Neurospora* ATPase by size exclusion chromatography or by glycerol gradient centrifugation revealed that these subunits formed a complex of apparent molecular mass 440 kDa (Bowman *et al.*, 1989). The information obtained from the dissociation studies of V-type ATPases suggests that these enzymes contain a peripheral complex consisting of five to six different subunit types, similar to the F_1 domain of F-type ATPases. However this complex has not yet been obtained in an active form.

The subunit stoichiometry of the coated vesicle ATPase has been determined by quantitative amino acid analysis of the separated subunits (Arai *et al.*, 1988). The nine subunits of the coated vesicle enzyme are present in a stoichiometry of three copies each of the 73 kDa and 58 kDa subunits, six copies of the 17 kDa subunit, and one copy each of the 100 kDa, 40 kDa, 38 kDa, 34 kDa, 33 kDa and 19 kDa subunits. This stoichiometry predicts an overall complex molecular mass for this vacuolar H^+ -ATPases of 758 kDa (Arai *et al.*, 1988), significantly higher than the usually determined value of 400-600 kDa as derived by a variety of techniques, including sedimentation analysis, gel filtration and radiation inactivation (Percy *et al.*, 1985; Bowman *et al.*, 1986; Mandala & Taiz, 1986; Randall & Sze, 1986; Xie & Stone, 1986; Arai *et al.*, 1988). Therefore, while the technique used by Arai *et al.*, (1988) may well be accurate, the results obtained must be treated with caution until supported with other types of evidence. However, it is worth noting that the predicted stoichiometry of the

coated vesicle ATPase is very similar to that of the bacterial F-type ATPase, which contains eight subunits in the stoichiometry of F_1 ($\alpha_3\beta_3\gamma\delta\epsilon$), F_0 (ab_2c_6-12). In mammalian mitochondria the enzyme is more complex and its subunit composition and stoichiometry remain controversial. Although the subunit stoichiometries of F-type and V-type may well be similar, the H^+ /ATP stoichiometries for the two classes of enzymes are different, being 2 for V-type ATPases and 3 for F-type ATPases (Cross & Taiz, 1990).

The results of the topographical and dissociation studies together with the information obtained about the subunit stoichiometry of the coated vesicle ATPase has led to the formulation of a general structural model for the V-type ATPases as a whole (Fig. 1.7). This model illustrates the striking structural similarity between the V-type and F-type ATPases. Like the F-type enzymes, V-type ATPases consist of two distinct domains, a peripheral complex on the cytoplasmic side of the membrane and an integral membrane complex. The peripheral complex of V-type ATPases contains up to five different subunit types, of approximate molecular masses 70 kDa, 57 kDa, 41 kDa, 34 kDa and 33 kDa. There are three copies of each of the 70 kDa and 57 kDa subunits, which have both been shown to be nucleotide binding proteins (see section 1.2.3.5). Therefore this domain, like the F_1 portion of the F-type ATPases, is believed to be the catalytic complex of V-type ATPases. The integral membrane complex contains multiple copies of the 17 kDa subunit which are believed to form a proton channel. This is supported by evidence that covalent modification of the 17 kDa proteolipid by DCCD inhibits proton translocation through the ATPase complex (see section 1.2.3.5). The subunits of intermediate size (20-40 kDa) appear to form the 'stalk' region which connects the catalytic head group to the membrane bound proton channel and the role of the large (100-120 kDa) subunit is unknown.

Further evidence for the structural similarity between the V-type and F-type ATPases comes from the examination of vacuolar membranes from plant, animal and fungal sources by electron microscopy. Immunocytochemical and negative staining techniques have revealed the “head and stalk” like structure of V-type ATPases from chromaffin granules (Moriyama *et al.*, 1991), kidney epithelial cells (Brown *et al.*, 1987), plant vacuoles (Klink & Lüttge, 1991; Morre *et al.*, 1991; Taiz & Taiz, 1991; Ward & Sze, 1992) and fungal vacuoles (Bowman *et al.*, 1989). The striking structural homology that exists between V-type and F-type H⁺-ATPases suggest that they are derived from a common evolutionary ancestor (see section 1.3).

1.2.3.5. Subunit function

Numerous studies have shown that the 70 kDa subunits of V-type ATPases possess a nucleotide-binding site. The ATP-protectable labelling of the 70 kDa subunit by Nbf-Cl has been demonstrated for the vacuolar pumps from coated vesicles (Arai *et al.*, 1987); plant vacuoles (Mandala & Taiz, 1986; Randall & Sze, 1987); *Neurospora* (Bowman *et al.*, 1986) and yeast (Uchida *et al.*, 1986), while ATP protectable labelling of the same subunit with NEM has been reported in clathrin-coated vesicles (Arai *et al.*, 1987); chromaffin granules (Percy & Apps, 1986; Moriyama & Nelson, 1987) and *Neurospora* (Bowman *et al.*, 1986). The labelling of the 70 kDa subunit in a ATP-protectable fashion strongly suggests that this subunit possesses the catalytic site of the ATPase complex, although this evidence is not absolutely conclusive: since there appear to be several classes of nucleotide-binding site, it could be argued that the susceptible sulphyryl group is an important regulatory site.

The 57 kDa subunit has also been shown to contain a nucleotide-binding

site. This was demonstrated by the ATP-protectable labelling of the 57 kDa subunit from red beet with the photo-activated ATP analogue 3-O-(4-(benzoyl)benzoyl)adenosine-5'-triphosphate (BzATP) (Manolson *et al.*, 1985). Without photo-activation, BzATP was shown to be a potent reversible inhibitor of the red beet ATPase. However, BzATP was not a simple competitive inhibitor of the ATPase but caused the enzyme to display cooperative kinetics with respect to ATP concentration. This suggests that the nucleotide site on the 57 kDa subunit is distinct from the catalytic site and that this subunit may play a regulatory role.

The DCCD labelling of a 17 kDa protein has been observed for the vacuolar proton pumps from chromaffin granules (Sutton & Apps, 1981); coated vesicles (Arai *et al.*, 1987); plants (Manolson *et al.*, 1985; Mandala & Taiz, 1986; Randall & Sze, 1986; Matsuura-Endo *et al.*, 1990); *Neurospora* (Bowman, 1983) and yeast (Uchida *et al.*, 1985). The ability of DCCD to inhibit the proton-pumping activity of V-type ATPases (Mandala & Taiz, 1985a; Wang & Sze, 1985; Arai *et al.*, 1987; Sabolic *et al.*, 1988; Wieczorek *et al.*, 1989) indicates that the 17 kDa subunit forms part of the proton channel of the ATPase complex. This is supported by evidence that the purified 17 kDa subunit from the coated vesicle ATPase is able to support passive proton transport when reconstituted into liposomes (Sun *et al.*, 1987). The 17 kDa protein like the 8 kDa DCCD reactive subunit of F-type ATPase is extremely hydrophobic as indicated by its extraction with chloroform : methanol and its aminoacid composition (Sutton & Apps, 1981; Arai *et al.*, 1987). The degree of DCCD labelling of the ATPase complex at which proton translocation is totally abolished is equivalent to one molecule of DCCD binding one molecule of enzyme (Arai *et al.*, 1987). This level of DCCD labelling only labels about one sixth of the 17 kDa subunits. This suggests that the formation of a functional proton channel requires the

cooperative interaction of at least six copies of the 17 kDa polypeptide and that the channel can be blocked by the reaction of one molecule of DCCD with any one of these subunits. Similar results have been obtained with the DCCD binding protein of F-type ATPases, which suggest the proton channel is made up of six to twelve copies of the 8 kDa subunit.

The function of the intermediate size subunits (20-40 kDa) in V-type ATPases is not yet known. These subunits are believed to form the "stalk" region which connects the catalytic "head" group to the membrane bound proton channel. Therefore, they may play some role in coupling the ATP hydrolytic activity of the enzyme to proton translocation, although the sequence data that has so far been obtained for these subunits (Hirsch *et al.*, 1988; Wang *et al.*, 1988; Foury, 1990) gives no indication of their function and has no homology to anything else.

The role of the largest (100-120 kDa) subunit is not yet understood. If it is accepted that it is not present in all V-type ATPases, and that it is present at only one copy per molecule of holoenzyme in mammalian enzymes, then it is likely that it is asymmetrically-associated with the rest of the complex. It is not known whether this subunit has an important role in forming the H⁺-conducting channel: for the bacterial F-type ATPase, all three (a, b and c) subunits of F₀ are necessary for H⁺-translocation (Friedl, *et al.*, 1981) and it is possible that the 100 kDa subunit of V-type ATPases is the functional equivalent of the a and b subunits in F-type ATPases.

A remarkable finding with the coated-vesicle ATPase is that removal of the 116 kDa subunit changes the specificity for divalent cations, so that the depleted fragment shows Ca²⁺-ATPase activity (Xie & Stone, 1988). This Ca²⁺-dependent ATPase preparation is unable to support proton-translocation, which has led to speculation that the 116 kDa subunit may

play some role in coupling ATP hydrolysis to proton-translocation (Stone *et al.*, 1989).

The 116 kDa subunit from the coated vesicle ATPase (which is probably identical to that of the neurosecretory vesicle ATPase) has been sequenced (Perin *et al.*, 1991). It has a structure that is composed of two domains: a hydrophilic amino-terminal domain in which almost one-third of the amino acids are positively or negatively charged, and a hydrophobic carboxy-terminal region which contains at least six transmembrane regions and is composed of >50% hydrophobic amino acids. These structural characteristics of the 116 kDa polypeptide agree well with its proposed function in coupling ATP hydrolysis to proton movement through the intramembraneous proton channel. This subunit has also been shown to be glycosylated (Adachi *et al.*, 1990), probably at a site exposed on the luminal side of the membrane, suggesting it may be involved in regulation of the pump's activity.

1.3. The evolution of H⁺-ATPases

The vacuolar H⁺-ATPases from a variety of endomembrane organelles and different eukaryotic kingdoms have been shown to belong to a single class of proton pumps, on the basis of new inhibitor sensitivities and common structural features (Forgac, 1989). Immunological studies have shown that antibodies raised to the 57 kDa and 67 kDa subunits of the V-type ATPase from red beet cross-react with polypeptides of similar size from V-type ATPases associated with chromaffin granules, clathrin coated vesicles and yeast vacuolar membranes (Manolson *et al.*, 1989). The antiserum raised against the 57 kDa polypeptides of the red beet ATPase also reacted with subunit "a" of the H⁺-ATPase from the obligately anaerobic

bacterium *Clostridium pasteurianum* and to the α subunit of the H^+ -ATPase from *E. coli*. There was no cross-reactivity with chloroplast or mitochondrial ATPases. These results together with gene sequence information (Gogarten *et al.*, 1989), as well as other structural properties suggest that all V-type ATPases are derived from a common evolutionary ancestor, and may be evolutionarily related to the F-type ATPases.

1.3.1. Sequence homologies between V-type and F-type ATPases

The genes encoding the 70 kDa subunits of carrot (Zimniak *et al.*, 1988); *Neurospora* (Bowman *et al.*, 1988a) and yeast (Hirata *et al.*, 1990) and the 57 kDa subunits of *Arabidopsis* (Manolson *et al.*, 1988); human brain (Bernasconi *et al.*, 1990); human kidney (Südhof *et al.*, 1989); *Neurospora* (Bowman *et al.*, 1988b) and yeast (Nelson *et al.*, 1989) V-type ATPases have all been sequenced. Comparisons with the sequences of the α and β subunits of F-type ATPases have confirmed that these two classes of proton pumps are evolutionarily related. Both the 70 kDa and 57 kDa subunits are highly conserved. There is 62% sequence homology between the 70 kDa subunits of plants and fungi, while there is over 70% identity between 57 kDa subunits of *Neurospora*, yeast and *Arabidopsis*. The overall identity of the 70 and 57 kDa subunits of the vacuolar ATPases with the corresponding β and α subunits of F-type ATPases is relatively low, about 25%. However, alignment studies have revealed several regions of high homology between the sequences of V-type 70 kDa subunits and F-type β -subunits including a putative active site region (Zimniak *et al.*, 1988; Gogarten *et al.*, 1989).

The genes encoding the 17 kDa proteolipid subunit from the chromaffin granule (Mandel *et al.*, 1988) and oat root (Lai *et al.*, 1991) and yeast (Nelson & Nelson, 1989) have also been sequenced and show strong sequence homology. Hydropathy plots predicted that the 17 kDa subunit was made up of four transmembrane helices, of which the fourth was particularly conserved showing 80% sequence identity between the oat and bovine proteolipids. This region contained a glutamic acid residue which is the possible site for DCCD-labelling. Alignment of the vacuolar sequences with F-type 8 kDa subunit sequences showed significant sequence similarity at both the amino and carboxy terminal ends (Mandel *et al.*, 1988; Nelson & Nelson, 1989). It has been proposed that the proteolipids of F-type and V-type ATPases evolved from a common ancestral gene which underwent a duplication event to give rise to the V-type subunit (Mandel *et al.*, 1988; Nelson & Nelson, 1989; Gogarten *et al.*, 1989; Cross & Taiz, 1990). Other V-type ATPase subunits which have been sequenced are 31 kDa subunits from bovine kidney (Hirsch *et al.*, 1988) and yeast (Foury, 1990), a 32 kDa polypeptide from chromaffin granules (Wang *et al.*, 1988) and the 116 kDa subunit of clathrin coated vesicles (Perin *et al.*, 1991). None of these subunits shows any sequence homology with F-type ATPases.

1.3.2. The archaeobacterial H⁺-ATPase

The properties of the plasma membrane H⁺-ATPases of several archaeobacteria have been characterised (Mukohata & Yoshida, 1987; Nanba & Mukohata, 1987; Lübben & Schäfer, 1987; Stan-Lotter *et al.*, 1991). These studies showed that the archaeobacterial H⁺-ATPases resemble more closely the V-type ATPases than the F-type ATPases. For example, the H⁺-ATPases of *Halobacterium halobium* and *Sulfolobus acidocaldarius* are

insensitive to azide and inhibited by nitrate (Nanba & Mukohata, 1987; Lübben & Schäfer, 1987). The hydrophilic complex of the archaeobacterial H⁺-ATPases contains two subunits α and β of similar size to the 70 kDa and 57 kDa subunits of V-type ATPases. Both the α (64kDa) and β (54 kDa) subunits of *Sulfolobus acidocaldarius* have been sequenced (Denda *et al.*, 1988a,b). Comparison of these sequences with sequences from vacuolar H⁺-ATPases revealed that the archaeobacterial α and β subunits were approximately 50% identical with the V-type 70 kDa and 57 kDa subunits (Gogarten *et al.*, 1989). There was found to be only 25% sequence identity between archaeobacterial and F-type ATPases (Nelson & Taiz, 1989). This data together with information obtained from immunological cross-reactivity studies (Konishi *et al.*, 1990) shows that the archaeobacterial H⁺-ATPase is more closely related to the V-type ATPase than the F-type ATPase.

1.3.3. Evolutionary relationship between F-type, V-type and archaeobacterial ATPases

The F-type and V-type ATPases are clearly distinct but related families of H⁺-ATPases, which differ from the P-type ATPases in their structure and catalytic mechanism. The low level of sequence homology between V-type and F-type ATPases and the finding that antibodies raised against V-type ATPase subunits will cross-react with bacterial F-type ATPases but not the F-type ATPases of higher eukaryotes suggests that the divergence of the two enzyme families is an ancient event, preceding the appearance of eukaryotes.

The archaeobacterial ATPases show a higher degree of sequence homology, and immunological cross-reactivity to the V-type ATPases than to

the F-type ATPases. This suggests that the archaebacterial ATPase is much more closely related to the V-type ATPase than the F-type ATPase. The archaebacterial ATPases probably diverged from the V-type ATPases at a point after the divergence between V-type and F-type enzymes.

1.4. Project Aims

The principal aim of the project was to develop a method for the purification and reconstitution of the vacuolar H⁺-ATPase from the CAM plant *Kalanchoë daigremontiana*. This was an essential prerequisite for detailed study of the kinetics and mechanism of regulation of the enzyme. Purification of the ATPase would also enable the subunit composition of the enzyme to be determined.

Antibodies were to be raised to selected subunits of the plant ATPase in order to investigate the structural homology and functional similarities of this tonoplast ATPase with V-type ATPases from other sources, in particular the chromaffin granule H⁺-ATPase from bovine adrenal glands, one of the best-understood examples of the endomembrane class of H⁺-ATPases in animal cells.

CHAPTER 2

2. Materials and General Methods

Special methods, the use of which were restricted to particular parts of the work, are described in the relevant chapters.

2.1. Materials

Triton X-114 was obtained from Fluka AG, (Buchs, Switzerland). *n*-Octyl- β -glucoside, polyvinylpyrrolidone (PVP-40) and 9-aminoacridine were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Acrylamide and bisacrylamide were obtained from BDH (Poole, Dorset, U.K.). Polyacrylamide was obtained from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). Bio-Gel P6-DG was from Bio-Rad (Richmond, CA, U.S.A). Cholesterol was supplied by Boehringer (Lewes, Sussex, U.K.). Bovine spinal-cord phosphatidylcholine, egg phosphatidylcholine and phosphatidylserine were obtained from Lipid Products (Redhill, Surrey, U.K.). Butylated hydroxytoluene was obtained from Koch-Light Laboratories Ltd. (Colnbrook, Bucks, U.K.). Nitrocellulose (0.45 μ m pore size) was obtained from Schleicher & Schüll (Dassel, Germany). Bafilomycin A₁ was a gift from Dr K. Altendorf, University of Osnabrück, Osnabrück, Germany. Antiserum to phosphoenolpyruvate carboxylase was a gift from Dr P. Maier and Dr M. Kluge, Institut für Botanik, Technische Hochschule Darmstadt, Germany. Antiserum directed against the 120 kDa subunit of the chromaffin granule H⁺-ATPase was prepared by Ms. J.M. Percy in this Department.

2.2. Plant Material

Plants of *Kalanchoë daigremontiana* Hamet et Perrier de la Bathie were propagated vegetatively and grown in John Innes No. 3 potting compost in a heated glasshouse. Natural lighting was supplemented by mercury-vapour lamps (400 W MBF; Thorn EMI, London, U.K.) for 12 h daily. When five to eight months old, plants were transferred to a reverse-phase controlled-environment room, where they were illuminated by a combination of metal-halide fluorescent lamps (400 W MBIF/ BU; Thorn) and tungsten lamps (PAR 38 150 W Flood; General Electric Co., Wembley, Middx.,UK) for 12 h daily at a photosynthetic photon flux density (400-700 nm) of $300 \mu\text{mol}/\text{m}^2 \text{s}^{-1}$ at mid-plant height. Air temperature was maintained at 25°C (light)/ 14°C (dark), with a relative humidity of approx. 35% (light)/ 70% (dark). Plants were maintained in the controlled environment room for at least 2 to 3 days prior to experimentation.

2.3. Tonoplast Isolation

Tonoplast fractions were prepared from the mesophyll tissue of *Kalanchoë daigremontiana* according to published methods (Bremberger *et al.*, 1988; White & Smith, 1989) with minor modifications. The leaf midrib and margins were removed and the mesophyll tissue was homogenised in a blender in a medium (typically 100 ml medium to 60 g tissue) containing 450 mM mannitol, 10 mM EGTA, 2 mM DTT, 0.5% (w/v) polyvinylpyrrolidone (PVP-40), 100 mM Tricine adjusted to pH 8.0 with Tris base. The homogenate was filtered through two layers of cheesecloth and the filtrate centrifuged (10,000 rev/min, Beckman JA 14 rotor, $g_{av} = 9820$) for 15 min. The resulting

supernatant was layered over a 0.78 M sucrose cushion containing 5 mM Tricine-Tris (pH 8.0) and 2 mM DTT. The gradients were centrifuged (36,000 rev/min, Beckman Ti 45 rotor, $g_{av}=100,000$) for 1 h. Tonoplast vesicles were removed from the interface using a Pasteur pipette and diluted 1:1 (v/v) with a medium containing 150 mM mannitol, 25 mM Tricine-Tris (pH 8.0) and 2 mM DTT. The vesicles were then pelleted by centrifugation (36,000 rev/min, Beckman Ti 45 rotor) for 30 min, and finally resuspended in 150 mM sucrose, 10 mM Tricine-Tris (pH 8.0) and 2 mM DTT. All fractionation steps were performed at 4°C and the tonoplast preparation was stored at -20°C.

2.4. Purification of Triton X-114

Triton X-114 was purified by the method of Bordier (1981). Twenty grams of Triton X-114 and 16 mg butylated hydroxytoluene, dissolved in 1 ml ethanol, were added to 980 ml Tris-HCl (pH 7.4), 150 mM NaCl. The detergent was dissolved in the buffer at 0°C. Incubation of the clear solution at 30°C led to condensation of the detergent and overnight the mixture separated into a large aqueous phase depleted in detergent and a smaller detergent-rich phase. The aqueous phase was discarded and replaced by the same volume of 10 mM Tris-HCl (pH 7.4), 150 mM NaCl. Buffer and detergent phase were mixed at 0°C and the condensation was repeated a further two times at 30°C under the same conditions. The third and final detergent-rich phase was used as the Triton X-114 stock for all experiments involving the detergent.

2.5. Estimation of detergent concentration of Triton X-114

The detergent concentration of the Triton X-114 stock solution was determined by the method of Garewal (1973). The assay of Triton X-114 by this procedure is based on the reaction of ammonium cobalthiocyanate with the poly(ethylene oxide) groups of Triton X-114 to form a blue precipitate. This is extracted into ethylene dichloride (or dichloromethane) and the extract is scanned spectrophotometrically to determine the concentration of the detergent.

Triton X-114 (0-600 mg) made up to 300 μ l in 50% (v/v) aqueous ethanol, (diluted from a stock solution of 2 mg/ml Triton X-114) was used for the standard curve. The sample to be assayed was diluted 1:100 in 50% ethanol and several dilutions were made for estimation of detergent concentration. The final volume of each dilution was 300 μ l. A 400 μ l aliquot of cobalthiocyanate reagent (17.8 g ammonium thiocyanate, 2.8 g cobalt nitrate hexahydrate, made up to 100 ml with water) was mixed with each sample and then left for 5 min at room temperature for colour development. Dichloromethane (1.5 ml) was then added, and each sample was vortexed for 2 min. Separation of the two phases was induced by centrifugation at 3000 rev/min in a bench centrifuge ($g_{av} = 1500$). The lower solvent layer was scanned in glass cuvettes using a Pye-Unicam SP1800 Split Beam spectrophotometer with a detergent blank as a reference. Scan speed was 2 nm/s and recorder speed 1 cm/min. The difference in absorbance between 622 and 688 nm was plotted against detergent concentration to generate a standard curve from which the detergent concentration in the sample was calculated.

2.6. Measurement of ATPase hydrolytic activity

Hydrolysis of ATP was measured at 37°C by a coupled spectrophotometric assay which measured the oxidation of NADH to NAD⁺ by a decrease in absorbance at 340 nm using a Pye-Unicam SP1800 Split Beam Spectrophotometer. The standard assay medium (1 ml) had the following composition: 2 mM ATP, 10 mM MgSO₄, 1 mM phosphoenolpyruvate, 0.2 mM NADH, 50 mM Hepes/KOH buffer (pH 7.4), lactate dehydrogenase (3.6 units/ml) and pyruvate kinase (3.0 units/ml).

2.7. Measurement of ATPase proton-pumping activity

Proton translocation activity of the reconstituted ATPase was measured at 30°C by recording the quenching of 9-aminoacridine fluorescence in a Perkin-Elmer 3000 fluorimeter, with excitation and emission wavelengths of 420 nm and 480 nm, respectively. The standard assay medium (0.5 ml) contained 0.3 M sucrose, 10 mM Hepes/NaOH buffer (pH 7.4), 1 mM ATP, 1 mM MgSO₄, 1.0 μM 9-aminoacridine, 0.36 μM valinomycin and reconstituted ATPase (usually about 5 μg protein). The initial rate of fluorescence quenching was shown to be directly proportional to the amount of ATPase protein in the assay and was taken to be directly proportional to the rate of H⁺-translocation (see Chapter 3).

2.8. Protein determination

The protein concentration of samples was determined either by the method of Bradford (1976) or by a modification of the Folin-Lowry method (Peterson, 1977).

2.8.1. The Bradford Assay

Bovine serum albumin was used as the standard, its concentration being checked by its absorbance at 280 nm (for a 1% soln. $A_{280} = 6.6$). Samples of 0 -10 μg BSA made up to 100 μl with distilled water were used for the standard curve. Several dilutions were made of the sample to be assayed. To all samples 1 ml of the Bradford reagent (50 $\mu\text{g/ml}$ Serva Blue G in 8.5% (v/v) orthophosphoric acid, 5% (v/v) ethanol) was added. The samples were then vortexed and left at room temperature for approximately 30 min for the colour to develop. The samples were then vortexed again to break up dye-protein aggregates and the sample absorbance at 595 nm was measured using a Cecil spectrophotometer, with a zero protein sample as blank.

2.8.2. The modified Folin-Lowry Assay

Duplicate samples of 0, 10, 20, 30, 40 and 60 μg BSA made up to 400 μl with distilled water were used for the standard curve. The sample to be assayed (containing between 5 and 60 μg of protein) was made up to 400 μl with distilled water. Forty microlitres of 0.15% (w/v) deoxycholate were added to the sample which was then mixed and allowed to stand for 10 min

at room temperature. Then 40 μ l of 72% (w/v) trichloro-acetic acid was added to the sample, which was then mixed and allowed to stand for a further 5 min. The protein present in the sample was precipitated and was pelleted by centrifugation in a microfuge for 10 min. The supernatant was discarded and the protein pellet was resuspended in 400 μ l distilled water.

Four hundred microlitres of reagent A (0.025% (w/v) CTC, 2.5% (w/v) SDS in 0.2 M NaOH) was added to the sample, mixed by vortexing and allowed to stand for 10 min at room temperature, to solubilize the precipitated protein. Finally 200 μ l of reagent B (Folin-Ciocalteu reagent diluted sixfold with distilled water) was added to the sample and mixed. After 30 min the absorbance of the sample at 750 nm was recorded using a Cecil spectrophotometer.

2.9. Gel electrophoresis

Polyacrylamide gel electrophoresis was usually performed using slab gels containing 10% (w/v) acrylamide (where indicated 12%, 15% straight gels or 8-15% exponential gradient gels were utilised). The ratio of acrylamide to N,N' methylene bis-acrylamide was 37.5:1 by weight. The gel system was taken from the method of Laemmli (1970), with SDS in the separating and stacking gels and 2-mercaptoethanol or dithiothreitol in the sample buffer. Acrylamide and bis-acrylamide used in the gels were of electrophoresis grade (BDH). Polyacrylamide (mol. wt. about 200,000) was added to strengthen the gel.

2.9.1. Gel preparation and pouring

Glass gel plates (18 x 14 cm) were cleaned with methanol and then clipped together with three PVC spacers (1.5 mm thick) on either side and along the bottom. One of the gel plates had 13 x 2 cm slot at the top. The sides and bottom were then sealed with a 1.5% (w/v) solution of molten agar. "Mini-gels" (8 x 8 cm, 0.75 cm thick) were also run; in this case the sides were clipped together and only the bottom was sealed with 1.5% (w/v) molten agar.

The separating buffer had the following composition: 0.375 M Tris-HCl (pH 8.8), 2 mM EDTA, 0.1% (w/v) SDS, 0.5% (w/v) polyacrylamide, 10% (w/v) acrylamide/ 0.27% (w/v) bisacrylamide, 0.05% (w/v) ammonium persulphate. Polymerisation of the gel was initiated by addition of 0.12% (v/v) TEMED prior to pouring.

The gel was poured to within 3 cm of the top of the gel plates to allow room for the stacking buffer, and water-saturated butan-2-ol was layered on top of the gel to give an even surface. After polymerisation of the separating gel, the butan-2-ol was rinsed off with distilled water and the stacking gel was applied.

The stacking gel had the following composition: 0.125 M Tris-HCl (pH 6.5), 2 mM EDTA, 0.1% (w/v) SDS, 4.5% (w/v) acrylamide/ 0.12% (w/v) bisacrylamide, 0.5% (w/v) polyacrylamide, 0.156% (w/v) ammonium persulphate. Polymerisation was started by the addition of 0.125% (v/v) TEMED and the mixture was poured on the top of the separating gel. Before the stacking gel set, a gel comb was inserted between the tops of the glass plates to allow the formation of wells for sample loading.

2.9.2. Sample preparation

Protein samples were dissolved in sample buffer containing 0.05 M Tris-HCl (pH 6.5), 5% (w/v) SDS, 2 mM EDTA, 10% (w/v) glycerol. 2-Mercaptoethanol (0.05%, v/v) or 10 mM DTT, and bromophenol blue (0.005%, v/v) were then added.

2.9.3. Running the gel

Once the stacking gel had polymerised, the gel comb and bottom spacer were removed from the cassette. Paraffin wax was applied to the top half of the slotted glass plate to enable a seal to be formed as the cassette was positioned on the gel tank. The gel tanks were then filled with electrode buffer (0.05 M Tris, 0.38 M glycine, 0.1% (w/v) SDS, 2 mM EDTA) and loose acrylamide was removed from the sample wells using a needle and syringe containing electrode buffer. Any bubbles at the bottom of the gel were also dislodged. Samples were loaded into the sample wells using a Hamilton syringe and gels were run overnight at a constant potential of 60-80 V.

Mini-gels were clipped to a Hoefer "Tall Mighty Small" slab gel apparatus and the comb was removed after the electrode buffer was poured. No loose polyacrylamide or air bubbles had to be dislodged, the bottom of the mini-gel remaining sealed with agar. Samples were loaded as above and running was normally at a constant current of 20 mA for about 2 h.

2.9.4. Fixing and staining the gel

When the dye front had reached the bottom of the gel, the tank was disconnected from the power-pack and the gel was removed. the glass

plates were separated and the gel placed in fixing solution (10% (v/v) acetic acid, 20% (v/v) methanol) for about 15-30 min, with gentle agitation. The gel was then drained and stained with 0.125% (w/v) Serva Blue R, 50% (v/v) methanol, 7.5% (v/v) acetic acid for 5-10 min. Destaining was achieved by pouring out the dye and soaking the gel in destaining solution (10% (v/v) methanol, 7% (v/v) acetic acid) in a shaking water bath. A piece of polyurethane foam was included to accelerate the destaining procedure by binding the dye.

2.9.5. Silver staining

The method of Wray *et al.* (1981) was followed. The gel was run and fixed as above. After fixing the gel was soaked in 50% (v/v) methanol for three 1 h periods with 5 min rinses with water inbetween. After this the gel was rinsed again with water and silver solution was then added. The silver solution was prepared as follows: 0.4 g of silver nitrate in 2 ml of water were added dropwise on a solution containing 21 ml of 0.36% (w/v) NaOH and 1.4 ml of 14.8 M ammonium hydroxide and the resulting solution was made up to 100 ml. After 5 min agitating in the silver solution, the gel was again rinsed with water and then developed with about 150 ml of 0.05% (w/v) citric acid, 0.0175% (w/v) formaldehyde. The gel was soaked in the developing solution until the protein bands were visualised, after which development was stopped with gel-fixing solution (see above).

2.10. Western blotting technique

2.10.1. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets

The protocol was taken from Towbin *et al.* (1979). Polyacrylamide gels were removed from the gel plates and placed in a 'sandwich' consisting of a support, scouring pads, filter paper and a nitrocellulose sheet. The transfer cassette was assembled in a tray containing transfer buffer (20 mM Na_2HPO_4 , 0.02% (w/v) SDS, 20% (v/v) methanol). With the cathode grid of the cassette at the bottom, the 'sandwich' was constructed in the following order: scouring pad, filter paper, polyacrylamide gel, nitrocellulose sheet, filter paper and scouring pad. Air bubbles were excluded by submerging the layers in buffer. The cassette was secured by clips and lifted into the transfer tank, transfer buffer being circulated by means of a pump. The proteins were then transferred for 2 h with a current of 0.8 A.

2.10.2. Antibody decoration of blots and visualisation of labelled proteins

After transfer the nitrocellulose blot was incubated with 0.5% (v/v) Tween 20 in 10 mM Tris-HCl (pH 7.4), 0.15 M NaCl for 1h to block the remaining protein binding sites. Once blocked, the blot was then incubated with the primary antibody (usually a rabbit polyclonal) which had been diluted in horse serum buffer (5% (v/v) heat inactivated horse serum, 25 mg/ml BSA, 0.5 mg/ml NaN_3 in 20 mM Tris-HCl (pH 7.2), 0.9% (w/v) NaCl) for 1.5-2 h. The blot was then given three 10 min washes with 0.05% (v/v) Tween 20 in 10 mM Tris-HCl (pH 7.4), 0.15 M NaCl to remove any non-specifically bound

primary antibody. After washing the blot was incubated with the secondary antibody, horse-radish peroxidase conjugated goat anti-rabbit F_{ab} fragments diluted 1:4000 into 5% (v/v) sheep serum in 10 mM Tris-HCl (pH 7.4), 0.15 M NaCl. The blot was given a further three 10 min washes with 0.05% (v/v) Tween 20 in 10 mM Tris-HCl (pH 7.4), 0.15 M NaCl to remove any non-specifically bound secondary antibody before being developed using the Amersham ECL system.

Alternatively, the labelled proteins could be detected using the biotin-streptavidin system. Blocking and decoration of the blot with primary antibody was performed as described above. The blot was then incubated with the secondary antibody, biotinylated goat anti-rabbit F_{ab} fragments diluted 1:200 into 5% (v/v) sheep serum in 10 mM Tris-HCl (pH 7.4), 0.15 M NaCl, for 2 h. The blot was then given three 10 min washes with 0.05% Tween 20 in 10 mM Tris-HCl (pH 7.4), 0.15 M NaCl, before being incubated with ¹²⁵I-streptavidin (10 cps/μl) in 10 mM Tris-HCl (pH 7.4), 0.15 M NaCl, for 1 h. The blot was given a further three 10 min washes with 0.05% Tween 20 in 10 mM Tris-HCl (pH 7.4), 0.15 M NaCl, before being dried and autoradiographed.

2.11. Raising antibodies to selected subunits of the tonoplast ATPase

Rabbit polyclonal antibodies were raised to the 57 kDa and 72 kDa subunits of the *Kalanchoë daigremontiana* tonoplast ATPase. Isolation of these subunits for immunisation was carried out as follows. About 2.5 mg of partially purified ATPase was run on a 8 -15% gradient gel. The gel was fixed, and stained with Coomassie brilliant blue. The strips of gel corresponding to the 57 kDa and 72 kDa subunits were cut out and these

proteins were removed from the gel by electro-elution.

Once sufficient quantities of the 57 kDa and 72 kDa subunits had been purified, four rabbits (two for each subunit) were immunised. The purified protein was mixed with Freund's complete adjuvant and administered by multiple site intradermal injections into the back of each rabbit.

Approximately 150-250 μ g of protein was used to immunise each rabbit.

Booster injections were given to the rabbits four weeks after the initial immunisation and every four weeks subsequently. Booster injections consisted of approximately 75-150 μ g purified protein mixed with Freund's incomplete adjuvant, and were administered by subcutaneous injection into the scruff of the neck of the rabbit. A total of three booster injections were given to each rabbit before the animals were terminally bled. The antisera obtained were aliquoted and stored at -20°C for later use.

CHAPTER 3

3. Purification and reconstitution of the tonoplast ATPase from *Kalanchoë daigremontiana*

3.1. Introduction

One of the principal aims of the project was to develop a method for purifying the *Kalanchoë* tonoplast ATPase. Published procedures for the purification of solubilized plant V-type ATPases usually involve either glycerol (or sucrose) density gradient centrifugation and/or column chromatography, and typically yield a product of specific activity 2.0 - 6.0 $\mu\text{mol}/\text{min}$ per mg protein (Mandala & Taiz, 1985*b*; Manolson *et al.*, 1985; Randall & Sze, 1986; Bremberger *et al.*, 1988; Matsuura-Endo *et al.*, Kasamo *et al.*, 1991). However, a specific activity of 20 - 25 $\mu\text{mol}/\text{min}$ per mg protein has been reported for the purified tonoplast ATPase of red beet (*Beta vulgaris* L) (Parry *et al.*, 1989).

These established methods for the purification of tonoplast ATPases are lengthy (up to 40 h). However, a rapid procedure for the purification and reconstitution of the V-type ATPase from bovine chromaffin granules (Pérez-Castiñeira & Apps, 1990) has been developed, involving the fractionation of membrane proteins with the detergent Triton X-114. This approach produced H^+ -pumping vesicles of high specific ATPase activity from native membranes within 2 h.

Purification of the tonoplast ATPase by HPLC and Triton X-114 fractionation was investigated to determine which approach was most suitable in the development of a protocol for the purification and reconstitution of the enzyme. Having developed a method for purifying and reconstituting the ATPase, it was then possible to study the subunit

composition of the enzyme complex and perform kinetic experiments on the reconstituted enzyme, in which both ATP hydrolysis and H^+ -translocation were measured (see Chapter 4).

3.2. Methods

3.2.1. HPLC purification of the tonoplast ATPase

Tonoplast membranes were thawed and sedimented (100,000 rev/min, Beckman TL 100.3 rotor, $g_{av} = 340,000$, 10 min). The membranes were then solubilised in 1.67% (w/v) MEGA-8, 20 mM Hepes/KOH (pH 7.6), 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, 0.02% (w/v) NaN_3 , to a final protein concentration of 2 mg/ml. The insoluble material was pelleted by centrifugation as described above. The supernatant was removed and loaded onto a Gilson G4000 SWXL size exclusion column, equilibrated with 0.1% (w/v) MEGA-8, 20 mM Hepes/KOH (pH 7.6), 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, 0.02% (w/v) NaN_3 . The flow rate through the column was 1 ml/min and the eluate was collected as 0.5 ml fractions.

3.2.2. Purification and reconstitution of the H^+ -ATPase

Tonoplast membranes were thawed and sedimented (100,000 rev/min, Beckman TL-100.3 rotor, $g_{av} = 340,000$, 10 min). All subsequent operations were performed at 0°C. The membrane pellet was resuspended to a final protein concentration of 2.0 mg/ml, using gentle homogenisation in a glass homogeniser. The resuspending buffer was 10 mM Hepes/KOH (pH.7.6), 0.15 M KCl, 1 mM EDTA, 2 mM DTT, containing 6.2 mg/ml Triton X-114.

Triton-insoluble material was collected by centrifugation as described above, washed by homogenisation in the same buffer containing Triton X-114 (6.2 mg/ml), and sedimented again. It was then resuspended in half the original volume of buffer, containing *n*-octyl β -glucoside (68 mM), phosphatidylcholine (2.7 mg/ml), phosphatidylserine (5.3 mg/ml) and cholesterol (2.0 mg/ml); the solution was then centrifuged and the clear supernatant carefully removed from the pellet. This supernatant was the solubilized, partially purified ATPase.

Aliquots of 0.2 ml of this solution were loaded onto 1 ml columns of Bio-Gel P6-DG that had been equilibrated with 10 mM Hepes/KOH (pH7.6), 0.15 M KCl, 2 mM DTT, 1 mM EDTA, 10% (v/v) methanol, and packed by centrifugation in a bench centrifuge (1 min, 1400 rpm; $g_{av} = 180$). The column eluates were collected by centrifugation under the same conditions, and stored on ice. These proteoliposomes were used for studying ATP-hydrolytic and proton-pumping activities.

Further purification of the tonoplast ATPase resolubilised after Triton X-114 fractionation was performed by centrifugation on glycerol gradients. Gradients (5 ml) were of 5-15% (w/v) glycerol in 0.15 M KCl, 10 mM Hepes-KOH (pH 7.6), 0.1 mM EDTA, 1 mM DTT, 10% (v/v) methanol, 35 mM *n*-octyl β -glucoside, 2.7 mg/ml phosphatidylcholine, 5.3 mg/ml phosphatidylserine and 2.0 mg/ml cholesterol. Aliquots of 0.4 ml of the solubilised ATPase were layered onto these gradients, which were centrifuged for 5 h at 45,000 rev/min (Beckman SW50.1 rotor; $g_{av} = 190,000$). After centrifugation, the gradients were fractionated into 0.5 ml samples, which were assayed for ATPase activity and analysed by SDS-polyacrylamide gel electrophoresis.

3.2.3. ^{14}C -DCCD labelling of tonoplast ATPase

Tonoplast membranes (2 mg/ml), reconstituted ATPase vesicles (0.5 mg/ml) and the fractions from the peak of ATPase activity on glycerol gradients were labelled with ^{14}C -DCCD (1.85 TBq/ μmol) by incubation of 100 μl aliquots of the samples with 11 kBq of ^{14}C -DCCD for 3 h at room temperature. After separation by electrophoresis, fluorography of the gels was carried out using 1 M sodium salicylate.

3.3. Development of a procedure for the purification and reconstitution of the tonoplast ATPase from *Kalanchoë daigremontiana*

One of the main aims of this project was to develop a method for the purification and reconstitution of the *Kalanchoë* tonoplast ATPase. A twin track approach was adopted, in which purification of the ATPase by HPLC and Triton X-114 fractionation were assessed in parallel. The most suitable method was then chosen for the development of a purification and reconstitution protocol for the ATPase.

3.3.1. Purification of the ATPase by HPLC

Before any samples could be loaded onto an HPLC column, the conditions for solubilisation of the tonoplast membranes had to be determined. Two non-ionic detergents octanoyl-*N*-methylglucamide (MEGA-8) and polyoxyethylene 9-lauryl ether (C_{12}E_9) were assessed for their ability to solubilise ATPase activity from the tonoplast preparation. Tonoplast

membranes were solubilised in solutions containing 0 to 2% (w/v) of these two detergents, to a final protein concentration of 2 mg/ml. The solubilised membranes were centrifuged to remove any insoluble material and then assayed for ATPase activity (Fig. 3.1). In general, membranes solubilised in MEGA-8 were found to have much higher ATPase activity than those solubilised in $C_{12}E_9$. There were two notable exceptions to this: membranes solubilised in 0.33% (w/v) and 1% (w/v) $C_{12}E_9$ had greater ATPase activity than membranes solubilised in the same concentrations of MEGA-8. However, these two cases run counter to the trend and should be treated with caution. Ignoring the erratic results obtained with $C_{12}E_9$, the highest ATPase activity was obtained when tonoplast membranes were solubilised in 1.67% (w/v) MEGA-8. Tonoplast membranes were solubilised in 1.67% (w/v) MEGA-8 for all subsequent experiments involving HPLC to purify the ATPase complex (see below).

It was found that addition of methanol or glycerol to a final concentration of 10% (v/v), to tonoplast membranes which had been solubilised in 1.67% (w/v) MEGA-8 (as described above) enhanced the ATPase activity of the enzyme (see Table 3.1). In the light of this observation 10% (v/v) glycerol was added to the column buffer used for HPLC.

Vacuolar ATPases are of relatively large molecular mass (400-500 kDa), so purification of the solubilised enzyme was attempted by size exclusion chromatography on a Gilson G4000 SWXL HPLC column, equilibrated with buffer containing 0.1% (w/v) MEGA-8 and 10% (v/v) glycerol (Fig. 3.2a). Recoveries of ATPase activity from the column were good with the total ATPase activity of the eluted fractions accounting for 68% of the activity loaded onto the column. Analysis of the eluted fractions by SDS-polyacrylamide gel electrophoresis showed that there was a large amount of protein in those fractions with highest ATPase activity. However, the size

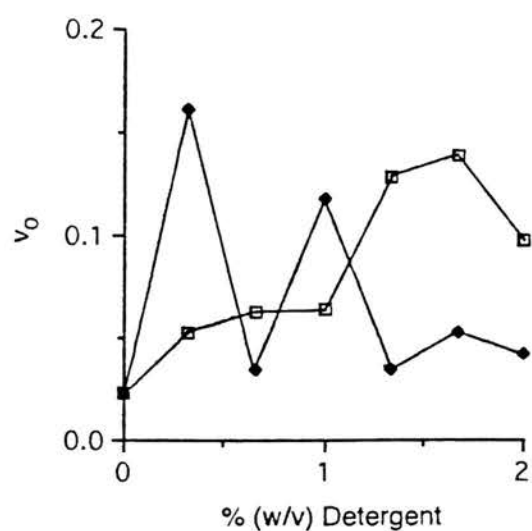


Fig. 3.1. Solubilization of the tonoplast ATPase. Tonoplast membranes were solubilised with either C₁₂E₉ (●) or MEGA-8 (◻), over a 0 to 2% (w/v) concentration range of these two detergents, and to a final protein concentration of 2 mg/ml. The solubilised membranes were centrifuged to remove any insoluble material and then assayed for ATPase activity. Initial rates (v_0) are expressed as $\mu\text{mol ATP hydrolysed/ min/ ml}$.

Sample	Specific Activity (nmol/ min per mg)
Solubilized ATPase	187
Solubilized ATPase + 10% (v/v) glycerol	512
Solubilized ATPase + 10% (v/v) methanol	651

Table 3.1. Enhancement of the activity of the solubilized ATPase with glycerol and methanol. The data shown are the ATPase hydrolytic activities observed for the solubilised ATPase in the absence and presence of 10% (v/v) glycerol or methanol. The values shown were obtained from a single preparation. See section 3.2.1 for solubilisation conditions.

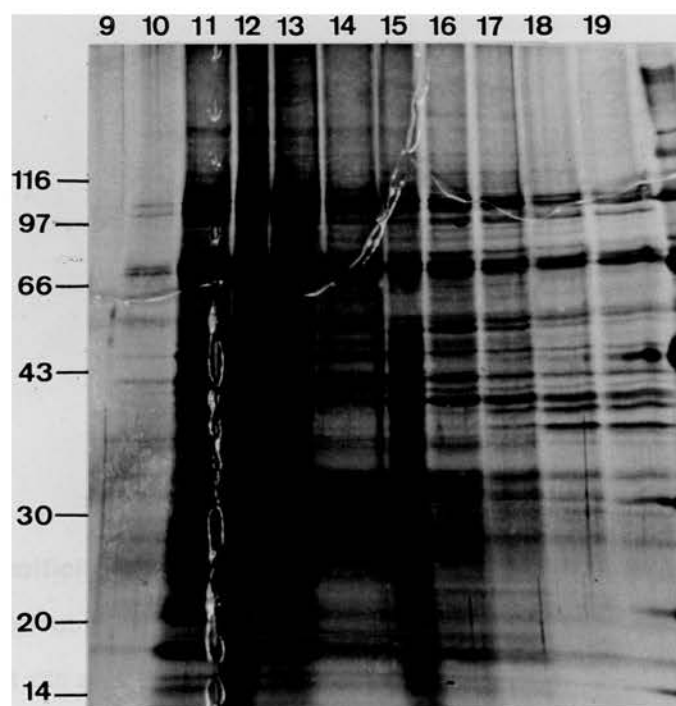
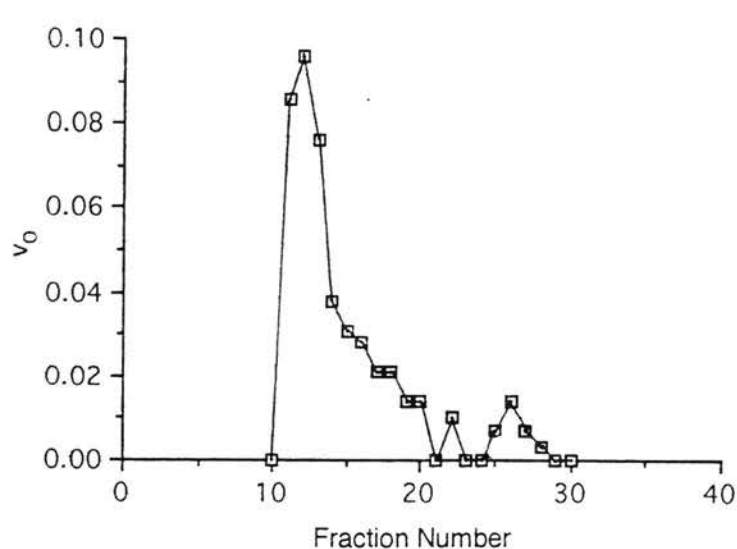
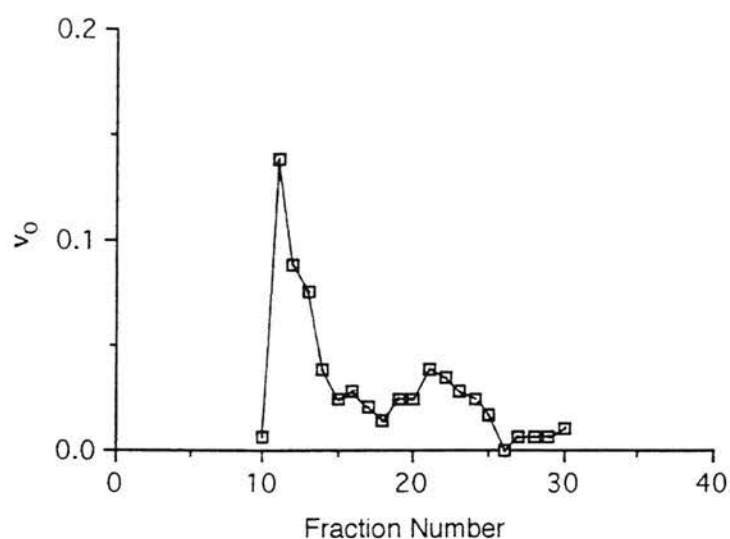
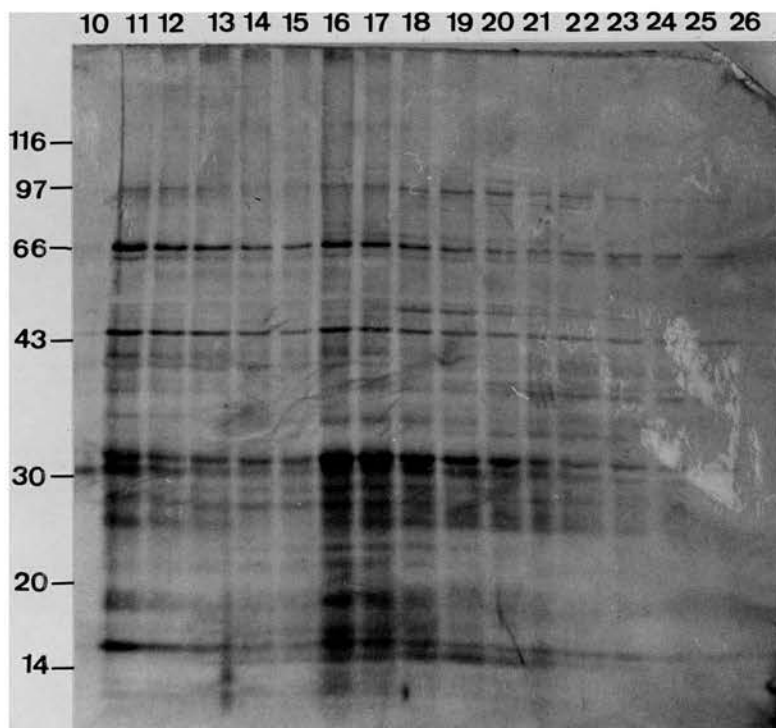


Fig. 3.2a-b. Chromatography of the solubilized tonoplast ATPase.

(a) Chromatography of tonoplast membranes solubilized in 1.67% (w/v) MEGA-8 on a Gilson G4000 SWXL size-exclusion column (see section 3.2.1, for conditions). One mg of solubilised protein was applied to the column. Column eluates were collected as 0.5 ml fractions which were assayed for ATPase activity. Initial rates (v_0) are expressed as $\mu\text{mol ATP hydrolysed/ min/ ml}$. (b) 8-15% polyacrylamide/ SDS gel of fractions 9-19 from column run shown in (a). Figures on the left of the gel indicate the positions of molecular mass standards (kDa).



c



d

Fig. 3.2c-d. Chromatography of the solubilized tonoplast ATPase.

(c) Chromatography of 0.8 mg of solubilized tonoplast protein on a G4000 SWXL column, under the same conditions as (a) with the addition of 100 mM KCl to the column buffer. Column eluates were collected as 0.5 ml fractions which were assayed for ATPase activity. Initial rates (v_0) are expressed as $\mu\text{mol ATP hydrolysed/ min/ ml}$. (d) 8-15% polyacrylamide/ SDS gel of fractions 10-26 from column run shown in (c). Figures on the left of the gel indicate the positions of molecular mass standards (kDa).

separation was poor suggesting that the proteins in the sample had aggregated (Fig. 3.2b). To overcome the problem of protein aggregation 100 mM KCl was added to the column buffer. Chromatography of the solubilised tonoplast under these conditions resulted in 56% of the ATPase activity loaded on to the column being recovered in the eluted fractions. The activity profile (Fig. 3.2c) is similar to that obtained in the absence of KCl in the column buffer (Fig. 3.2a). However analysis of the eluted fractions by SDS-polyacrylamide gel electrophoresis (Fig. 3.2d) indicated that the H⁺-ATPase complex had undergone partial dissociation during purification. This approach was subsequently abandoned in favour of the Triton X-114 procedure.

3.3.2. Purification of the ATPase by fractionation of tonoplast membranes with the detergent Triton X-114

3.3.2.1. Initial conditions for the purification of the tonoplast ATPase

To determine the optimal Triton X-114 concentration for the purification of the ATPase, tonoplast membranes were solubilised (to a final protein concentration of 2 mg/ml) in buffer containing varying concentrations of detergent, ranging from 0.6% (w/v) to 1.6 (w/v) Triton X-114. For each detergent concentration, the Triton-insoluble material was pelleted by centrifugation, then the precipitates were resuspended in buffer and assayed for ATPase activity. The detergent concentration which produced the precipitate with the highest ATPase activity was 0.6% (w/v) Triton X-114 (Fig. 3.3).

Having determined the Triton X-114 concentration for fractionation of the

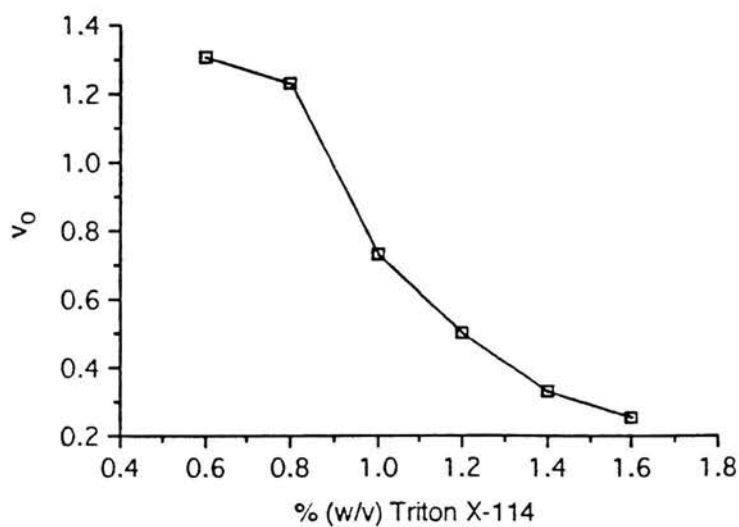


Fig. 3.3. Fractionation of tonoplast membranes with Triton X-114. Tonoplast membranes were solubilised to a final protein concentration of 2 mg/ml with varying concentrations of detergent, ranging from 0.6 to 1.6% (w/v) Triton X-114. The Triton insoluble material was pelleted by centrifugation, the precipitate was then resuspended in buffer and assayed for ATPase activity. Initial rates (v_0) are expressed as $\mu\text{mol ATP hydrolysed/ min/ ml}$.

tonoplast membranes, the ATPase was then purified by the following method. Tonoplast membranes were solubilised in buffer containing 0.6% (w/v) Triton X-114 to a final protein concentration of 2 mg/ml. The Triton-insoluble material was pelleted and washed by homogenisation in the same buffer containing 0.6% (w/v) Triton X-114. This material was pelleted for a second time and then resuspended in half the original volume of buffer containing 20 mg/ml *n*-octyl glucoside. The solution was centrifuged and the clear supernatant carefully removed from the pellet. This was the solubilised, partially purified ATPase.

3.3.2.2. Determination of the lipid requirements for reconstitution

The ATPase was purified as described above and then 200 µl aliquots of the solubilised enzyme (0.24 mg/ml) were reconstituted (see section 3.2.2) with varying quantities of a lipid mixture containing only phosphatidylcholine (PC) and phosphatidylserine (PS) in the ratio 2:1 (by weight). The quantity of lipid ranged from 0.5 to 2.5 mg (Fig. 3.4). Optimal ATPase activity was observed with a lipid:protein ratio of 40:1 (equivalent to a total lipid concentration of 10 mg/ml). Higher ATPase activity could be achieved with a lipid:protein ratio of 50:1, but not all the lipid was solubilised at this concentration. Thus it was decided to use a total lipid concentration of 10 mg per ml of solubilised enzyme when reconstituting the ATPase.

Having determined how much lipid to use, it was then necessary to determine what lipid composition would give the best ATPase activity. It was decided to use just three lipids, PC, PS and cholesterol to make up the lipid mixture to be used for reconstitution. the presence of cholesterol in the lipid mixture was thought to be necessary to produce sealed vesicles, since it is known to enhance the mechanical stability of the lipid bilayer.

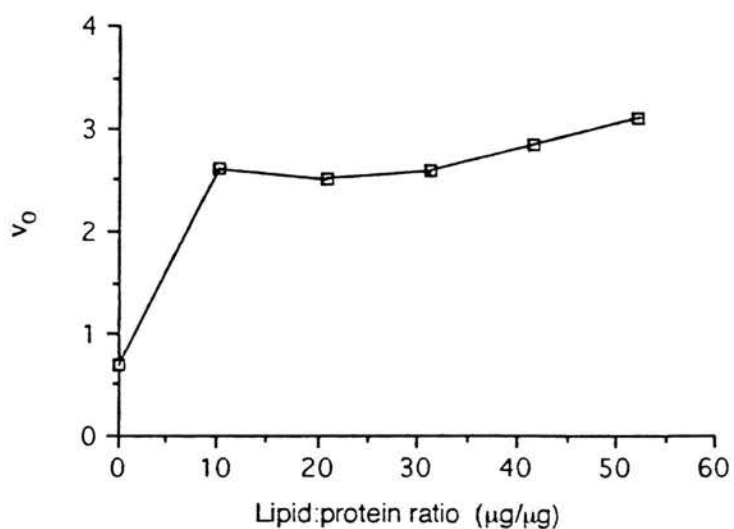


Fig. 3.4. Dependence of ATPase activity on the lipid:protein ratio. Aliquots of solubilised ATPase (0.24 mg protein/ ml) were reconstituted with differing amounts of a lipid mixture containing 2:1 PC:PS, by weight. The lipid:protein ratio was varied from 10:1 to 50:1, and for each ratio the ATPase activity of the reconstituted enzyme was assayed. Initial rates (v_0) are expressed as $\mu\text{mol ATP hydrolysed/ min/ mg}$.

The ratio of PC:PS was optimised (Fig. 3.5) by varying the ratio of PC:PS from 100% PC to 100% PS whilst keeping the total lipid concentration at 10 mg/ml. A ratio of 2:1 PC:PS was found to give the best results. Cholesterol was then added to the lipid mixture, the fraction by weight of cholesterol being varied from 0-25% while maintaining a PC:PS ratio of 2:1 (Fig. 3.6). Cholesterol appeared to have little effect on the ATPase activity of the reconstituted enzyme when present up to 15% of the total lipid mixture. Above 15% cholesterol ATPase activity progressively declined.

Analysis of the lipid composition of tonoplasts from mung bean (*Vigna radiata* L.) (Yoshida & Uemura, 1986) revealed that this membrane contained 18.2% free sterols. More recently it has been reported that the tonoplast membrane from *Kalanchoë daigremontiana* contains 21.4% free sterols (Haschke *et al.*, 1990). Sterols are structurally similar to cholesterol and therefore probably enhance the mechanical stability of the tonoplast membrane in much the same way that cholesterol strengthens mammalian membranes.

In order to produce sealed vesicles, with which to measure the proton-pumping activity of the reconstituted ATPase, it was believed that the concentration of cholesterol in the lipid mixture would have to be similar to the concentration of free sterols in the tonoplast membranes. Therefore a cholesterol concentration of 25% was chosen for the optimal lipid mixture. Thus the optimal lipid mixture for the reconstitution of the tonoplast ATPase was deduced to be 2:1:1 (by weight) PC:PS:Cholesterol. However, when assayed the reconstituted ATPase did not show any proton-translocating activity in liposomes of this lipid composition.

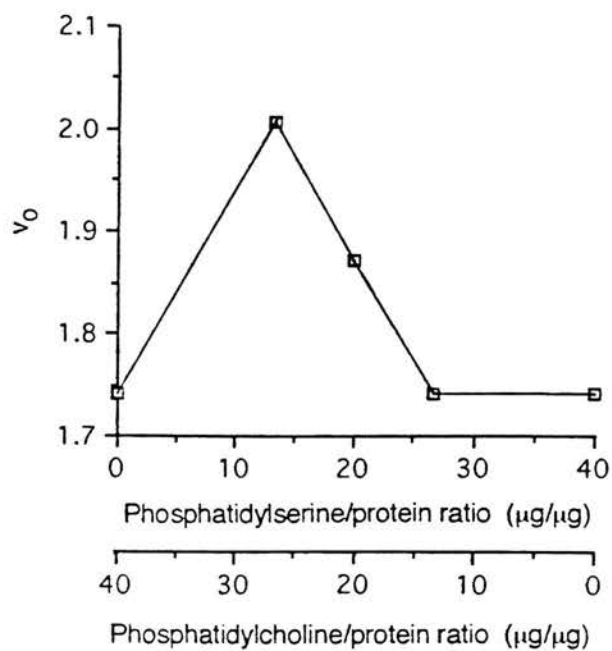


Fig. 3.5. Dependence of ATPase activity on the phosphatidylcholine/phosphatidylserine ratio. Total phospholipid content was 40 μg/μg of protein (this was equivalent to a total phospholipid concentration of 10 mg/ml). Initial rates (v_0) are expressed as μmol ATP hydrolysed/ min/ mg protein.

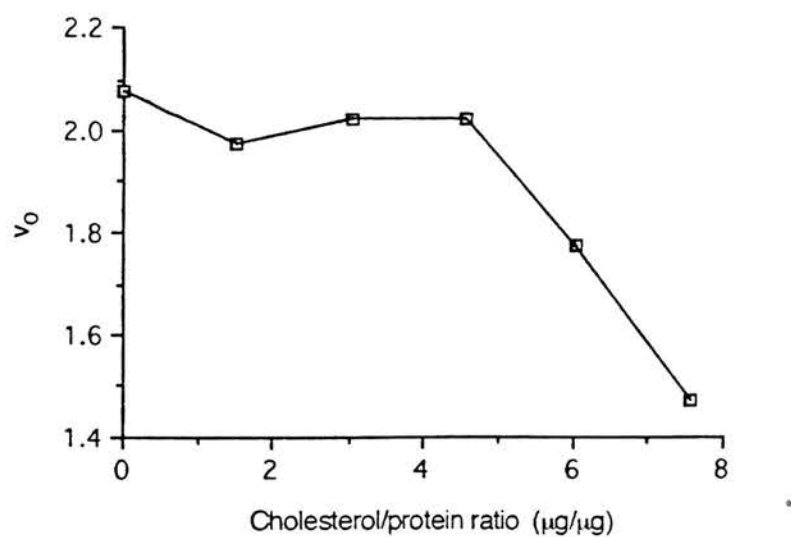


Fig. 3.6. Dependence of ATPase activity on the cholesterol/protein ratio.

Ratio of PC:PS was maintained at 2:1 while the cholesterol concentration was varied. Total phospholipid content was 30 $\mu\text{g}/\mu\text{g}$ of protein (this was equivalent to a total phospholipid concentration of 10 mg/ml). Initial rates (v_0) are expressed as μmol ATP hydrolysed/ min/ mg protein. No H^+ -translocation was observed with these liposomes.

3.3.2.3. Increasing the concentration of Triton X-114 used to purify the ATPase

Fractionation of tonoplast membranes with 0.6% (w/v) Triton X-114 did not produce proteoliposomes which had any proton pumping activity. It was therefore decided to try a higher detergent concentration in order to produce a cleaner enzyme preparation which would exhibit H⁺-translocating activity when reconstituted with commercial lipids. Tonoplast membranes (2 mg protein/ml) were fractionated with 0.8% (w/v), 1.0% (w/v) and 1.2% (w/v) Triton X-114. The ATPase was reconstituted into proteoliposomes using the previously determined lipid mixture of 2:1:1 (by weight) PC:PS:Cholesterol. The ATP-hydrolytic and proton-pumping activities observed for proteoliposomes prepared by fractionation with 0.8% (w/v), 1.0% (w/v) and 1.2% (w/v) Triton X-114 are shown in Table 3.2. From these data 1.0% (w/v) Triton X-114 appeared to be the optimal detergent concentration for reconstituting vesicles with both ATP hydrolytic and proton pumping activities.

3.3.2.4. Re-optimisation of lipid conditions for reconstitution

Having determined that 1.0% (w/v) Triton X-114 was the optimal detergent concentration for purification of the ATPase, it was decided to re-examine the lipid conditions required for ATP-hydrolytic and proton-pumping activities when reconstituting the partially purified ATPase into proteoliposomes.

Firstly, the ratio of PC:PS was optimised as before by altering the ratio of PC:PS from 100% PC to 100% PS while maintaining a total lipid concentration of 10 mg/ml. The stability of the reconstituted ATPase in proteoliposomes of differing PC/PS composition was also assessed by

[Triton X-114] (%)	Specific activity (nmol/ min per mg)	Initial rate of quenching (Fractional fluorescence quench/ min per μg protein)
0.8	614	0.024
1.0	708	0.038
1.2	556	nil

Table 3.2. Optimisation of Triton X-114 concentration for reconstitution of the tonoplast ATPase. The data shown are the ATPase hydrolytic and H^+ -translocation activities observed for the reconstituted ATPase when prepared with different concentrations of Triton X-114. For each detergent concentration, the values shown were obtained from a single preparation.

assaying the ATPase activity of these proteoliposomes over a three day period (Fig. 3.7). Proteoliposomes with a lipid composition of 1:1 PC:PS were found to be most stable. Therefore 1:1 PC:PS was taken as the optimal ratio for these two lipids when reconstituting the ATPase. The amount of cholesterol in the lipid mixture was optimised by increasing the concentration of cholesterol from 0 to 2.5 mg/ml while maintaining a total lipid concentration of 10 mg/ml and a 1:1 PC:PS ratio. Comparison of the activities observed for ATP hydrolysis (Fig. 3.8a) and H⁺-pumping (Fig. 3.8b) revealed that a cholesterol concentration of 2 mg/ml gave "optimal" activity.

Having found the optimal cholesterol concentration for the lipid mixture, the PC:PS ratio was re-examined to determine whether 1:1 PC:PS was the optimal PC:PS ratio in the presence of 2 mg/ml cholesterol. The ratio of PC:PS was varied from 1:0 to 0:1 in the presence of 2 mg/ml cholesterol to find the optimal mixture of PC, PS and cholesterol for both ATP-hydrolytic and proton-pumping activities. The total lipid concentration was maintained at 10 mg/ml. Comparison of the activities recorded for ATP hydrolysis (Fig. 3.9a) and proton-translocation (Fig. 3.9b) showed that a 1:2 ratio of PC:PS was optimal for both activities. Thus, the optimal lipid mixture for reconstituting the tonoplast ATPase was found to be 2.7 mg/ml PC, 5.3 mg/ml PS and 2 mg/ml cholesterol.

3.3.2.5. Problems with reconstituting the tonoplast ATPase

From the results described above, fractionation of tonoplast membranes with 1.0% (w/v) Triton X-114 followed by reconstitution of the ATPase into liposomes with lipid composition of 27:53:20 PC:PS:cholesterol, appeared to be the optimal conditions for purification and reconstitution. However, this

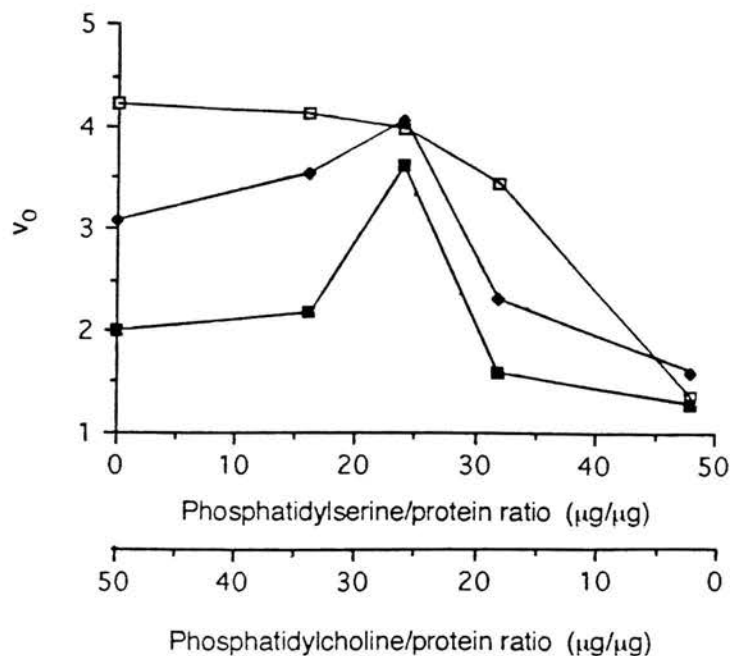


Fig. 3.7. Effect of the phosphatidylcholine/phosphatidylserine ratio on the stability of the reconstituted ATPase. Total phospholipid content was $48 \mu\text{g}/\mu\text{g}$ of protein (this was equivalent to a total phospholipid concentration of 10 mg/ml). ATPase activity was assayed 1 (\square), 2 (\blacklozenge) and 3 (\blacksquare) days after reconstitution. Initial rates (v_0) are expressed as $\mu\text{mol ATP hydrolysed/ min/ mg protein}$.

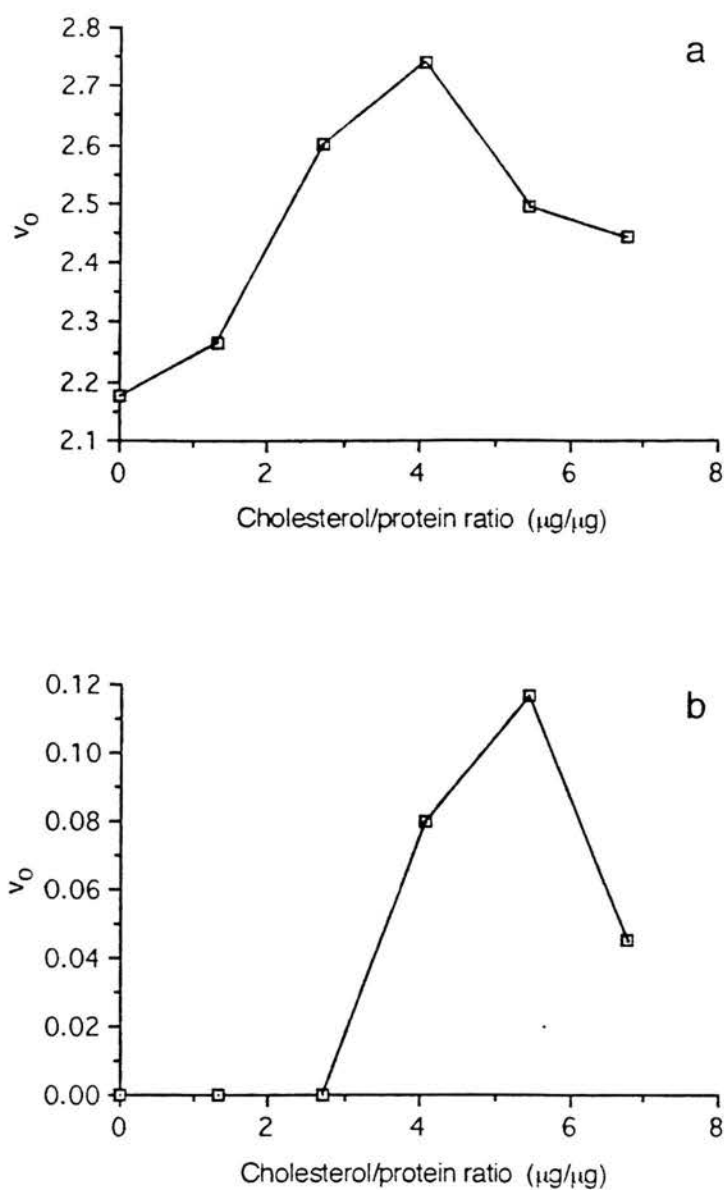


Fig. 3.8. Effect of varying the cholesterol/protein ratio on ATPase activity

(a) and H^+ -translocation (b). Ratio of PC:PS was maintained at 1:1 while the cholesterol concentration was varied. Total phospholipid content was $27 \mu g/\mu g$ of protein (this was equivalent to a total phospholipid concentration of 10 mg/ml). For ATPase activity, initial rates (v_0) are expressed as μmol ATP hydrolysed/ min/ mg protein. For H^+ -translocation, initial rates (v_0) are expressed as fractional fluorescence quench/ min per μg protein.

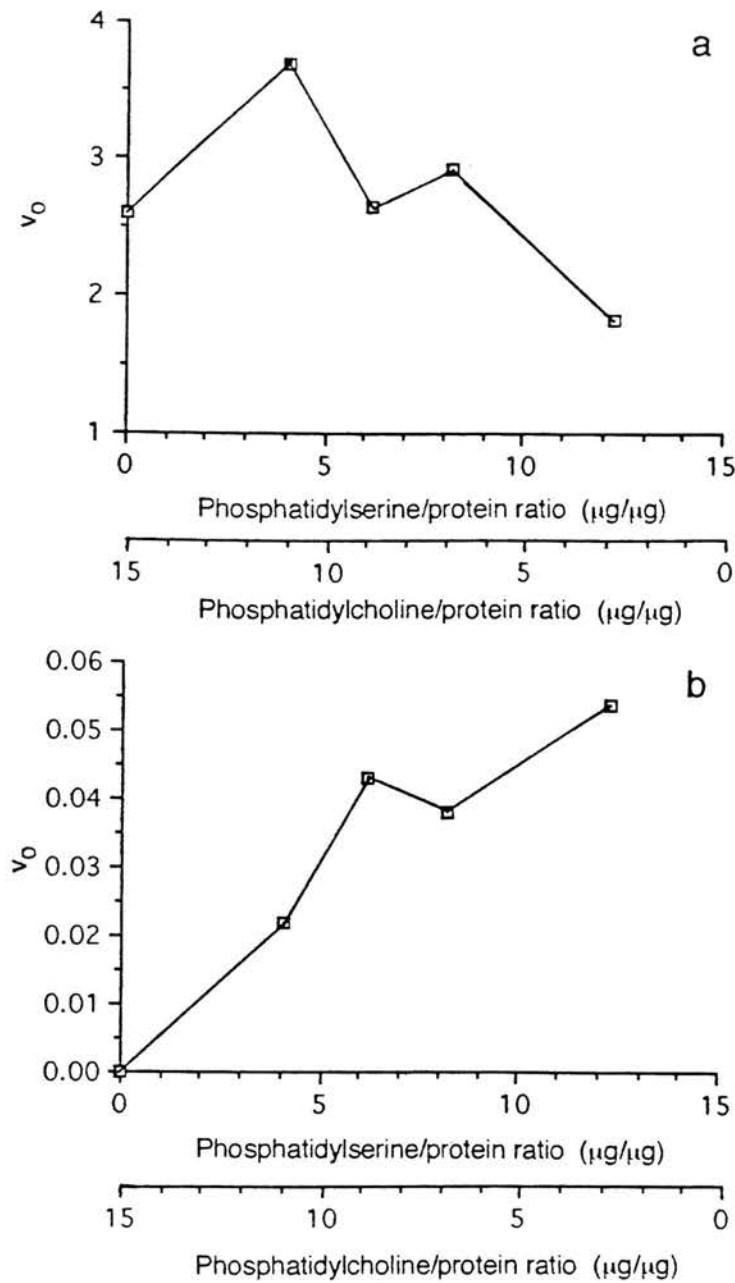


Fig. 3.9. Re-optimization of the phosphatidylcholine/phosphatidylserine ratio. Cholesterol/protein ratio was maintained at 3 $\mu g/\mu g$ (equivalent to a cholesterol concentration of 2 mg/ml) while the PC:PS ratio was varied. Total phospholipid content was 15 $\mu g/\mu g$ of protein (this was equivalent to a total phospholipid concentration of 10 mg/ml). The reconstituted ATPase was assayed for ATPase activity (a) and H^+ -translocation (b). For ATPase activity, initial rates (v_0) are expressed as μmol ATP hydrolysed/min/mg protein. For H^+ -translocation, initial rates (v_0) are expressed as fractional fluorescence quench/min per μg protein.

procedure was not always successful in producing proton-translocating proteoliposomes of high specific ATPase activity. The conditions used for solubilisation of the ATPase in *n*-octyl glucoside were found to be critically important for the successful reconstitution of the enzyme. By monitoring the ATPase activity of the sample at each stage of the purification procedure it was found that the ATPase was often rapidly inactivated after solubilisation in *n*-octyl glucoside.

Purification of the tonoplast ATPase by Triton X-114 fractionation presumably removes much of the lipid which would otherwise surround the enzyme complex in the membrane. The absence of this lipid when the partially purified ATPase was resolubilised with *n*-octyl glucoside could lead to the inactivation of the enzyme. It was found that active reconstituted ATPase was reliably obtained when solubilisation of the enzyme with *n*-octyl glucoside was performed in the presence of lipids already dissolved in the detergent, and this procedure was therefore adopted as standard. In the light of this finding the concentration of Triton X-114 used to fractionate the tonoplast membranes was once more optimised to see if a lower Triton concentration would be less damaging to the enzyme. The concentration of Triton X-114 was varied from 5.0-9.0 mg/ml. The ATP-hydrolytic and proton-pumping activities of the reconstituted ATPase were recorded for each Triton concentration used (Fig. 3.10). From this information the optimal concentration of Triton X-114 for the reconstitution of the ATPase was found to be 6.2 mg/ml. The finalised conditions for the purification and reconstitution of the *Kalanchoë* tonoplast ATPase are outlined in section 3.2.2 of this Chapter. These were found to be the optimal conditions for reproducibly producing proton-pumping proteoliposomes with high specific ATP-hydrolytic activity, and were used to produce the proteolipid vesicles used in kinetic studies (see Chapter 4).

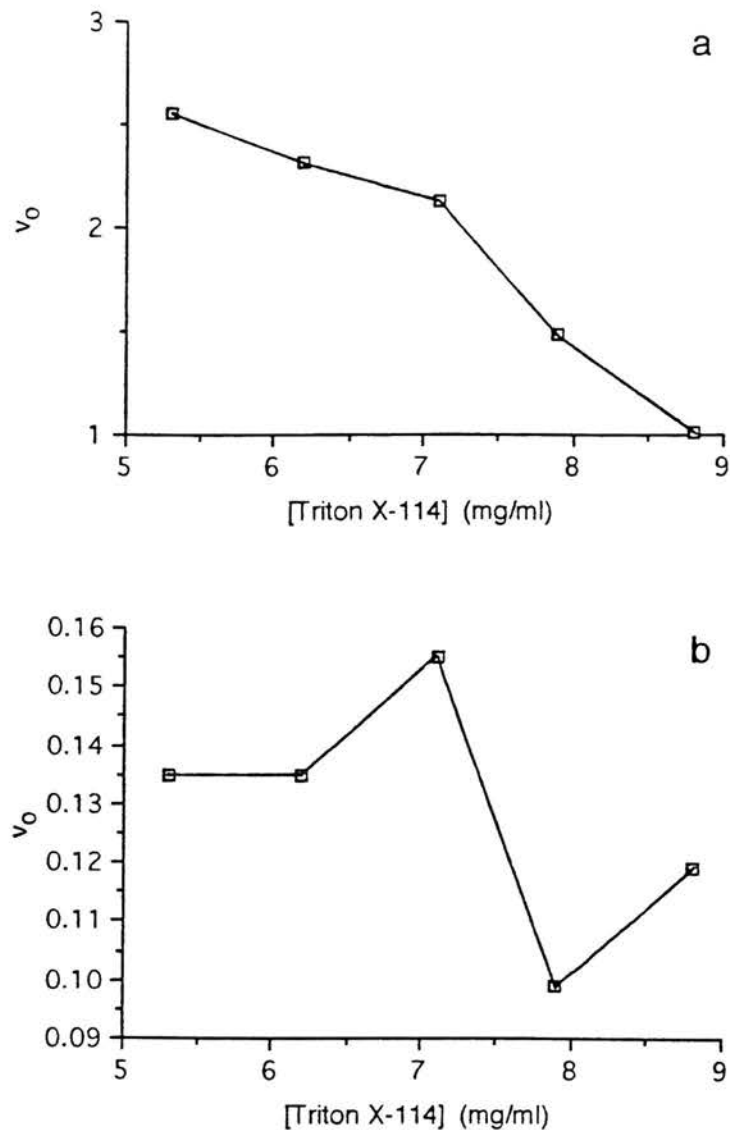


Fig. 3.10. Effect of varying the concentration of Triton X-114 on ATPase activity (a) and H⁺-translocation (b). Tonoplast membranes were solubilised to a final protein concentration of 2 mg/ml with varying concentrations of Triton X-114, ranging from 5.0 to 9.0 mg/ml detergent. The ATPase was reconstituted with a mixture PC, PS and cholesterol (27:53:20, by weight). The total phospholipid concentration was 10 mg/ml. For ATPase activity, initial rates (v_0) are expressed as $\mu\text{mol ATP hydrolysed/ min/ mg protein}$. For H⁺-translocation, initial rates (v_0) are expressed as fractional fluorescence quench/ min per $\mu\text{g protein}$.

3.3.2.6. Purification and reconstitution of the tonoplast ATPase

The purification and reconstitution of the tonoplast H⁺-ATPase is summarised in Table 3.3, which shows the data for a typical preparation and the average specific activities from four preparations. The final specific activity reported in Table 3.3 is that obtained on reconstitution with a mixture of phosphatidylcholine, phosphatidylserine and cholesterol in the ratio 27:53:20 by weight (see section 3.2). This lipid mixture was found to be optimal for measuring both the ATP-hydrolytic and proton-pumping activities of the enzyme and produced a threefold stimulation in ATPase activity on reconstitution of the solubilised ATPase into proteoliposomes. Similar stimulation was observed with the chromaffin granule H⁺-ATPase (Pérez-Castiñeira & Apps, 1990), although in this case the optimal lipid composition was quite different.

3.4. Subunit composition of the purified ATPase

A Coomassie-blue stained SDS-polyacrylamide gel of the ATPase at the various stages of purification is shown in Fig. 3.11. The reconstituted enzyme contains major polypeptides with molecular mass 72, 57, 48, 42 and 33 kDa; a 16 kDa subunit stains poorly, but was revealed by radioactive labelling (see below). Small amounts of a 100 kDa protein co-purified with the ATPase. Immunoblotting of the fractions shown in Fig. 3.11 identified this protein as the cytosolic enzyme *phosphoenol* pyruvate carboxylase (Fig. 3.12a). Furthermore, antibodies raised against the 120 kDa subunit of the bovine chromaffin granule H⁺-ATPase (Gillespie *et al.*, 1991) failed to recognise any antigen in the tonoplast ATPase (Fig. 3.12b).

Stage	Activity (nmol/ min per ml)	[Protein] (mg/ml)	Specific activity (nmol/ min per mg)	Recovery (%)
Membrane	1722	2.0	861 (1006±21)	(100)
Precipitate	1672	1.55	1079 (1449 ± 494)	72.9 (86.7 ± 13.0)
Washed precipitate	1371	1.28	1075 (1265 ± 357)	59.7 (56.8 ± 7.2)
Solubilised ATPase	568	0.59	963 (1131 ± 517)	16.5 (17.2 ± 8.0)
Reconstituted ATPase	1597	0.59	2707 (2897 ± 542)	46.4 (42.9 ± 8.9)

Table 3.3. Reconstitution of tonoplast ATPase. The data shown are for a typical preparation; values in parentheses are the means ± S.D. of specific activities and recoveries obtained in four separate preparations.

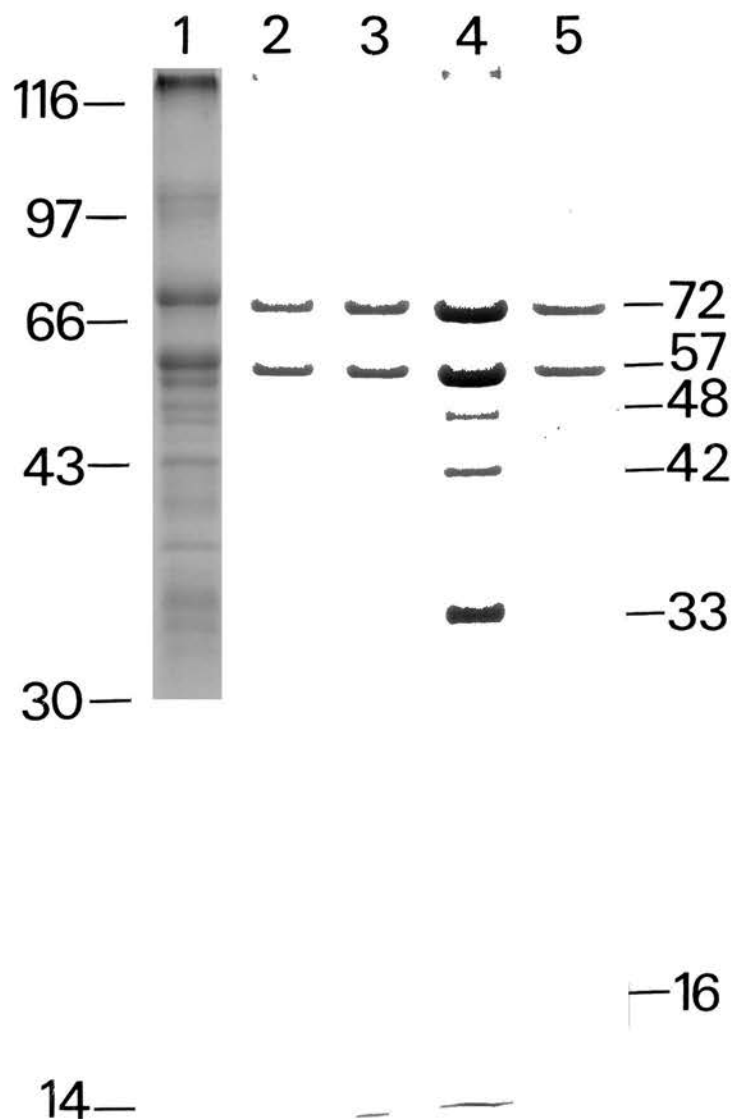


Fig 3.11. 12%-polyacrylamide/SDS gel of ATPase fractions at different stages of purification (see Table 3.3), stained with Coomassie blue. Track 1, tonoplast membranes; track 2, protein precipitated with Triton X-114; track 3, washed precipitate; track 4, resolubilised ATPase; track 5, reconstituted ATPase. Each track contained 20 µg of protein. Figures on the left indicate the positions of molecular mass standards; figures on the right show the apparent molecular masses of major polypeptides (kDa).

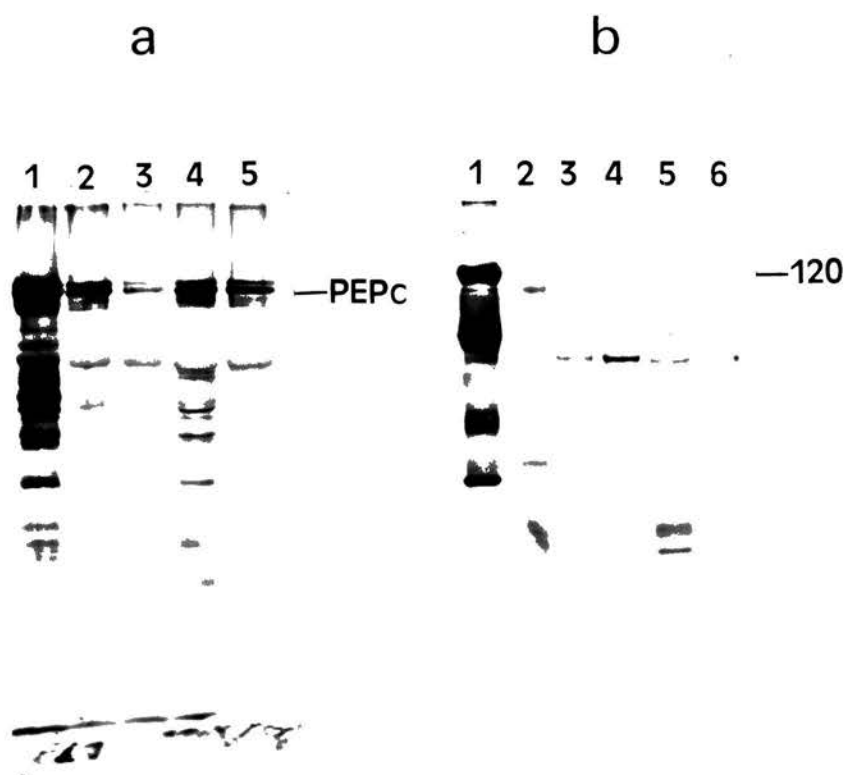


Fig. 3.12. Immunoblots of *Kalanchoë* ATPase fractions at different stages of purification. (a) track 1, tonoplast membranes; track 2, tonoplast protein precipitated with Triton X-114; track 3, washed precipitate; track 4, resolubilised tonoplast ATPase; track 5, reconstituted tonoplast ATPase. (b) track 1, Triton-insoluble fraction from chromaffin granule membranes; track 2, tonoplast membranes; track 3, tonoplast protein precipitated with Triton X-114; track 4, washed precipitate; track 5, resolubilised tonoplast ATPase; track 6, reconstituted tonoplast ATPase. Each track contained 20 μ g of protein (except track 1 of 3.12b, 10 μ g). The samples were subjected to SDS-PAGE using a mini-gel apparatus, transferred to nitrocellulose and probed with: (a) a 1:500 dilution of anti-phosphoenolpyruvate carboxylase serum; (b) a 1:200 dilution of chromaffin granule anti-120 kDa serum, followed by horse-radish peroxidase anti-rabbit goat IgG and visualisation using the ECL system.

The solubilised ATPase was further purified by centrifugation through glycerol gradients. This procedure removed some minor contaminants from the solubilised ATPase and the ATPase activity profile down the gradient is shown in Fig. 3.13, but the protein concentrations were too low for accurate measurement, so the specific activity could not be determined. A silver-stained polyacrylamide gel of the gradient fractions of highest ATPase activity is shown in Fig. 3.14a. These fractions contain the same polypeptides as the reconstituted enzyme vesicles (Fig. 3.11); silver-staining also revealed another polypeptide, of apparent molecular mass 39 kDa. The 16 kDa subunit (not seen in Fig. 3.14a which is a 10% polyacrylamide gel) was strongly labelled by ^{14}C -DCCD (Fig. 3.14b); some labelling of the 72 and 57 kDa subunits also occurred, although this was not seen in the unfractionated tonoplasts that were treated with ^{14}C -DCCD. In the reconstituted vesicles, most of the label was apparently incorporated into lipid.

3.5. The measurement of H^+ - translocation by the reconstituted ATPase

3.5.1. The use of fluorescent dyes for the measurement of H^+ -translocation

The proton-pumping activity of H^+ -translocating ATPases is usually measured using optical probes such as ACMA (9-amino-6-chloro-methoxy-acridine), 9-aminoacridine, or quinacrine (atebrin), which are primary amine derivatives of acridine (a detailed discussion of how these fluorescent dyes work is given in Chapter 1, section 1.1.3.4 and Fig. 1.2). The fluorescent

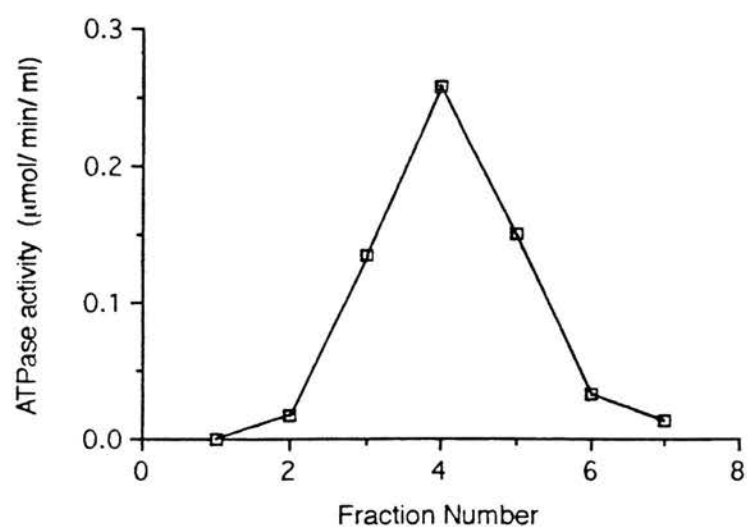


Fig. 3.13. Glycerol gradient centrifugation of the solubilised ATPase. ATPase activity of fractions collected from the gradient. The protein concentration of the fractions was too low to determine the specific activity of the samples.

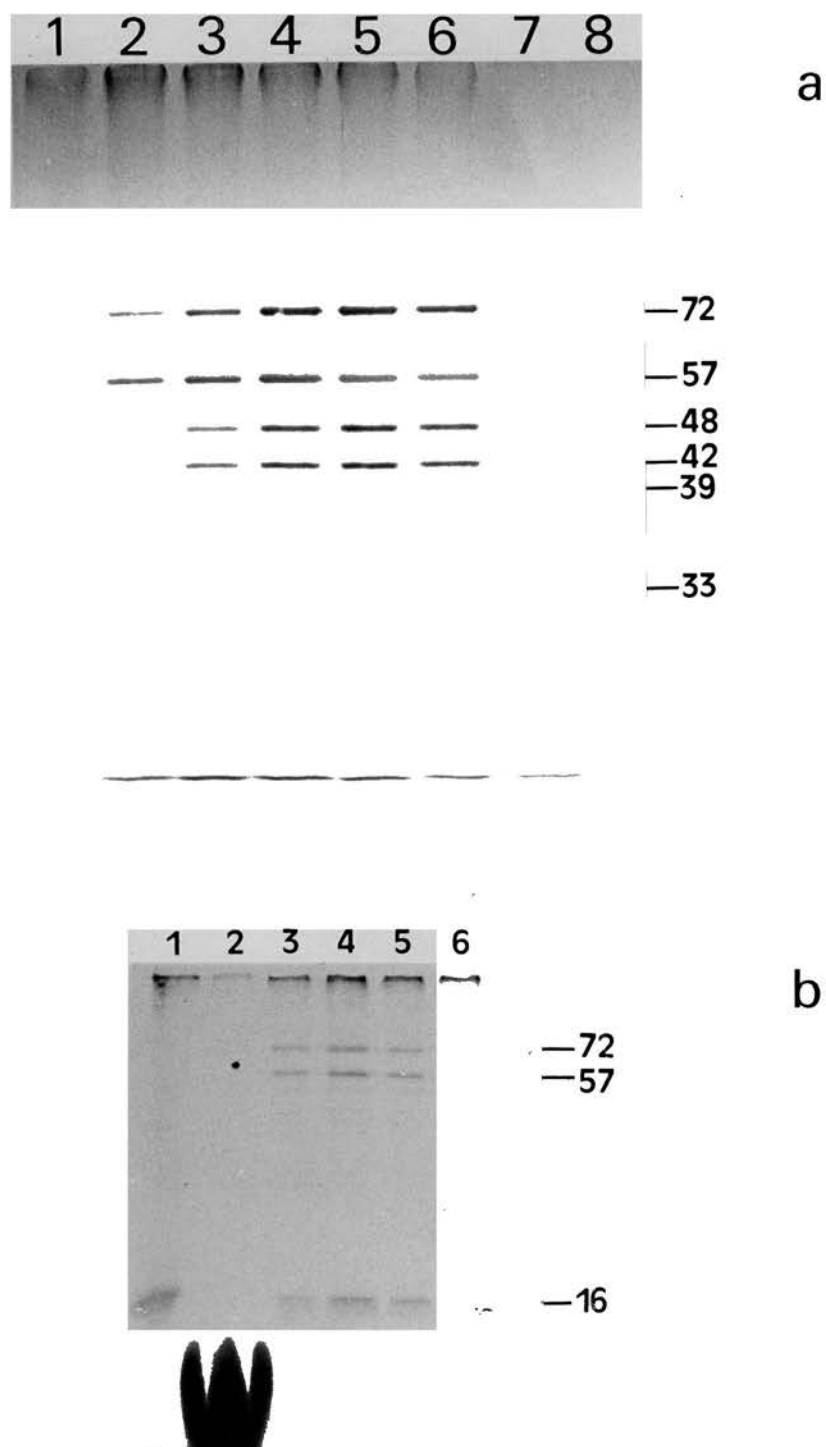


Fig. 3.14. Subunit composition of the purified ATPase. (a) 10%-polyacrylamide/SDS gel of fractions with ATPase activity on glycerol density-gradient centrifugation, stained with silver. Fraction 4 had the highest activity. (b) Autoradiograph of a 15%-polyacrylamide/SDS gel of fractions labelled with $[^{14}\text{C}]$ -DCCD. Track 1, tonoplast membranes; track 2, proteoliposomes; tracks 3-6, glycerol-gradient fractions. Figures on the right of each gel indicate apparent molecular masses (kDa) of subunits.

probes 9-aminoacridine and quinacrine have been used to measure ATP-dependent proton-translocation in plant tonoplast vesicles (Wang & Sze, 1985; Jochem & Lüttge, 1987; White & Smith, 1989), while ACMA has been successfully used to measure the H^+ -translocation activity of chromaffin granule "ghosts" (Percy *et al.*, 1985) and the reconstituted chromaffin granule ATPase (Pérez-Castiñeira & Apps, 1990). These three probes were assessed for their suitability for measuring proton-translocation by the reconstituted *Kalanchoë* tonoplast ATPase. Significant ATP-dependent fluorescence quenching was observed with both 9-aminoacridine (Fig. 3.15a,b) and quinacrine (Fig. 3.15c). However, 9-aminoacridine was found to be a more sensitive probe than quinacrine and was therefore chosen as the fluorescent probe for measurement of the H^+ -translocation activity of the reconstituted *Kalanchoë* ATPase. Typical traces of the quenching of 9-aminoacridine fluorescence obtained under different conditions are shown in Fig. 3.16. When ACMA was assessed for its ability to measure the H^+ -translocation of the reconstituted tonoplast ATPase, considerable fluorescence quenching was observed in the absence of ATP, upon addition of valinomycin (Fig. 3.15d). This effect made ACMA unsuitable for the measurement of the H^+ -translocation activity of the reconstituted ATPase. No such effects were observed with either 9-aminoacridine or quinacrine.

In order to confirm that the quenching of 9-aminoacridine fluorescence was a suitable method for measuring a pH difference across liposomal membranes a titration experiment was performed: 10 μ l of reconstituted ATPase were used to produce ATP-dependent quenching of 9-aminoacridine fluorescence, and once maximal quenching was achieved, the external pH of the solution was changed by addition of small aliquots of HCl,

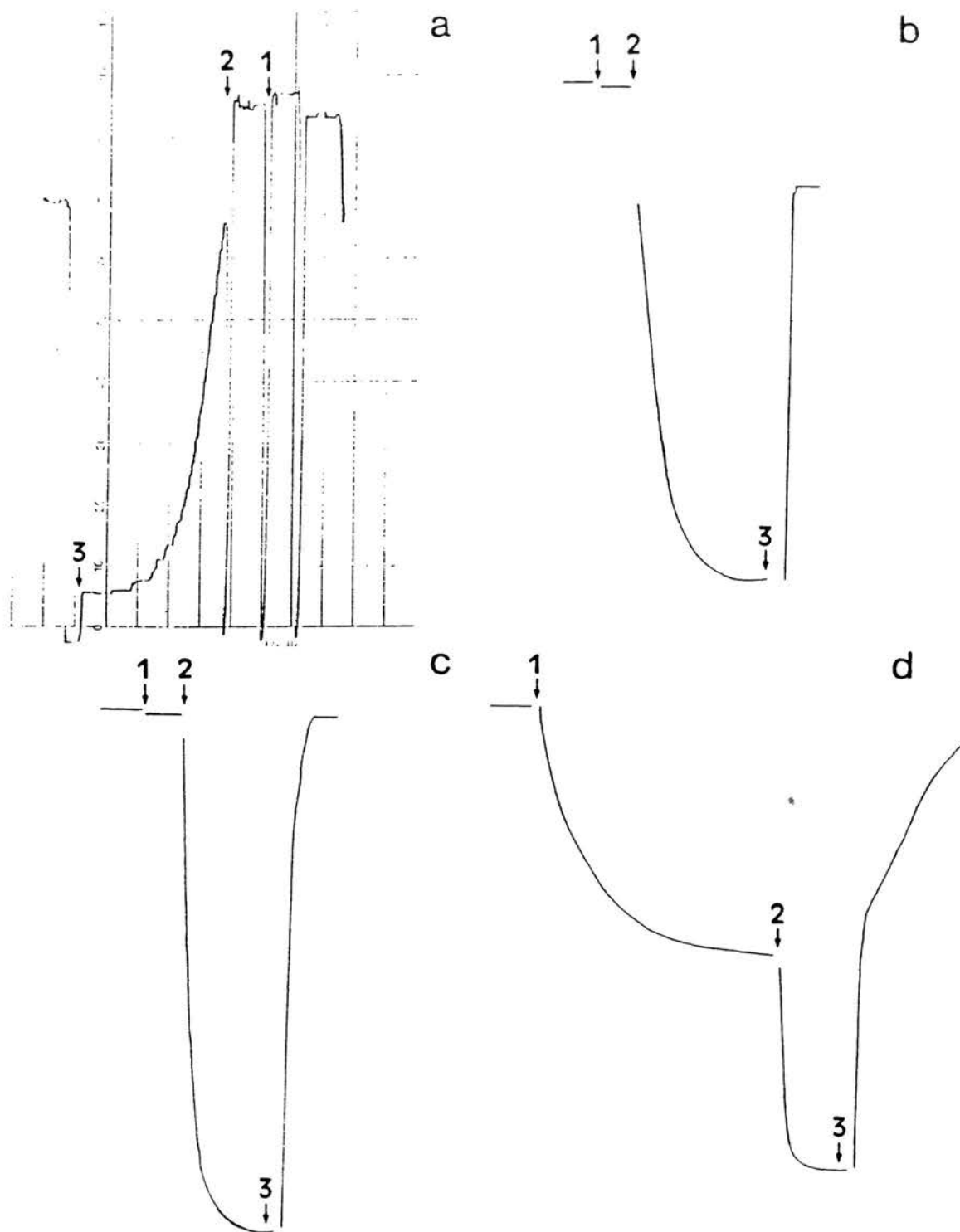


Fig. 3.15. The measurement of H^+ -translocation using 9-aminoacridine (a,b), quinacrine (c) and ACMA (d). Ten μ l of reconstituted ATPase (about 5 μ g) were incubated in assay medium (see Chapter 2, section 2.7). In Figs. 3.15a and 3.15b the concentration of 9-aminoacridine was 1.0 μ M. In Fig. 3.15c the concentration of quinacrine was 4.2 μ M. In Fig. 3.15d the concentration of ACMA was 1.0 μ M. In each case valinomycin (1), ATP (2) and nigericin (3) were added so that the final concentrations were 0.36 μ M, 1 mM and 0.67 μ M respectively. N.B. For convenience the fluorescence traces shown in Figs. 3.15b-d are copies of the original recordings. For comparison the original trace of 9-aminoacridine fluorescence quenching is shown in Fig. 3.15a.

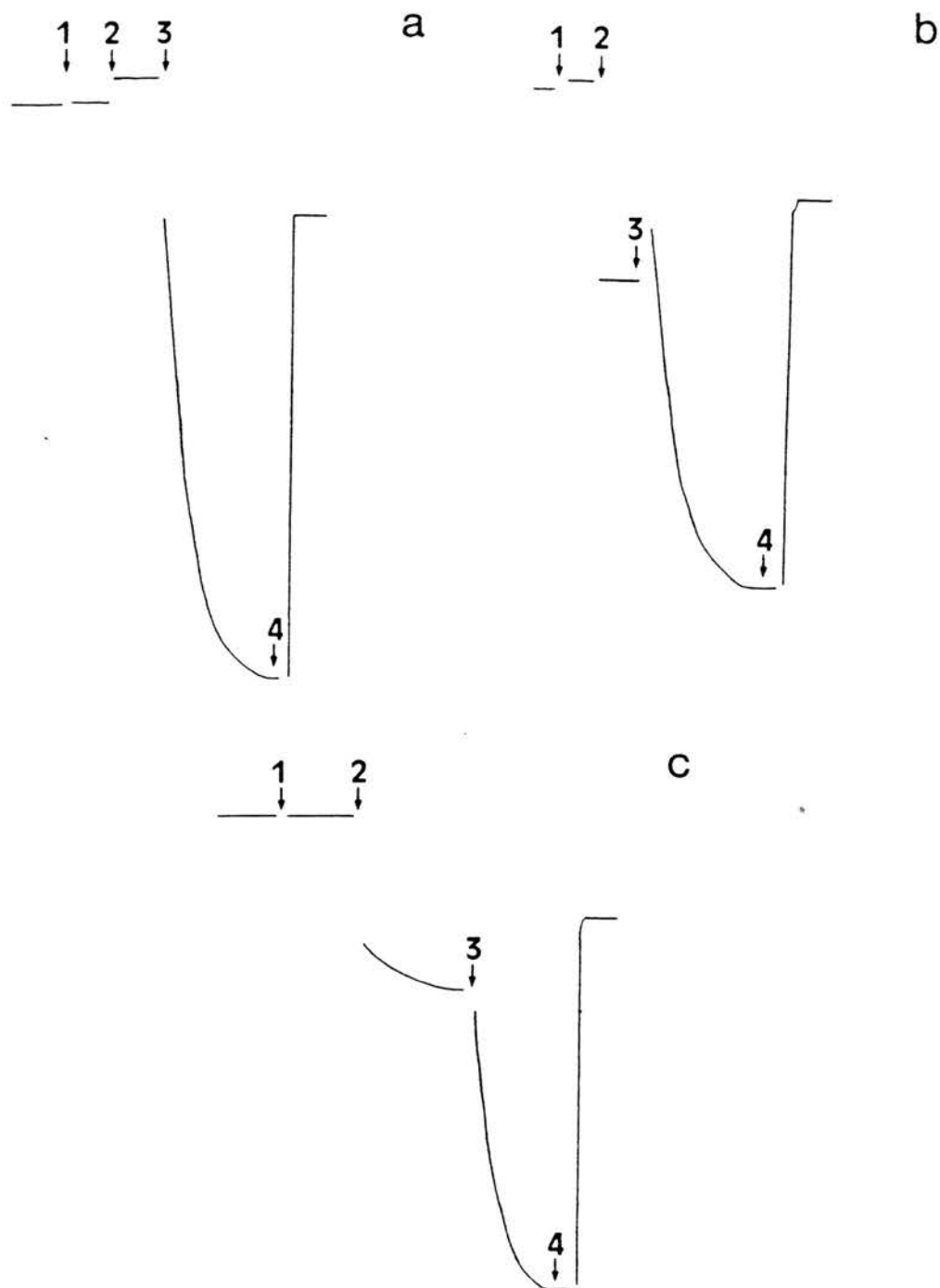


Fig. 3.16. Dependence of 9-aminoacridine quenching on the addition of (a) ATP, (b) Mg^{2+} and (c) valinomycin. Ten μ l of reconstituted ATPase (about 5 μ g) were incubated in assay medium (see Chapter 2, section 2.7). In Fig. 3.16a where indicated valinomycin (1), $MgSO_4$ (2), ATP (3) and nigericin (4) were added. In Fig 3.16b valinomycin (1), ATP (2), $MgSO_4$ (3) and nigericin (4) were added. In Fig. 3.16c $MgSO_4$ (1), ATP (2), valinomycin (3) and nigericin (4) were added. In each case valinomycin, $MgSO_4$, ATP and nigericin were added so that the final concentrations were 0.36 μ M, 1 mM, 1mM and 0.2 μ g/ml respectively.

which produced small increases in fluorescence as the extent of quenching (Q) was reduced by the decrease in the external pH (Fig. 3.17). When $\log [Q / (1-Q)]$ was plotted against external pH (pH_{out}) a straight line was produced, with a slope of 2.75 (Fig. 3.18). The response of 9-aminoacridine does not accord to the Schuldiner equation which predicts a slope of 1.0 (see Chapter 1, section 1.1.3.4 and Fig. 1.2). The reason for this non-ideal behaviour is unknown, but the response of this probe to changes in pH is linear and therefore it can still be considered suitable for measuring a transmembrane proton gradient. Quenching of 9-aminoacridine fluorescence was absolutely dependent on the addition of ATP (Fig. 3.16a), Mg^{2+} (Fig. 3.16b) and valinomycin (Fig. 3.16c).

3.5.2. Initial rate of fluorescence quenching as a measure of H^+ -translocation activity

The use of the initial rate of fluorescence quenching as a measure of H^+ -translocation activity is widely reported in the literature (Flatmark *et al.*, 1985; Grønberg & Flatmark, 1987; Gluck & Al-Awqati, 1984; Bennett & Spanswick, 1983). Gluck & Al-Awqati (1984) and Bennett & Spanswick (1983) have produced theoretical justifications that this rate is actually proportional to the rate of proton accumulation into a sealed compartment. For the reconstituted *Kalanchoë* ATPase, the initial rate of 9-aminoacridine quenching was shown to be directly proportional to the amount of protein present (Fig 3.19): in the absence of vesicles, the quench rate was zero. Thus, the initial rate of fluorescence quenching was taken as a direct measure of the H^+ -translocation activity of the protein.

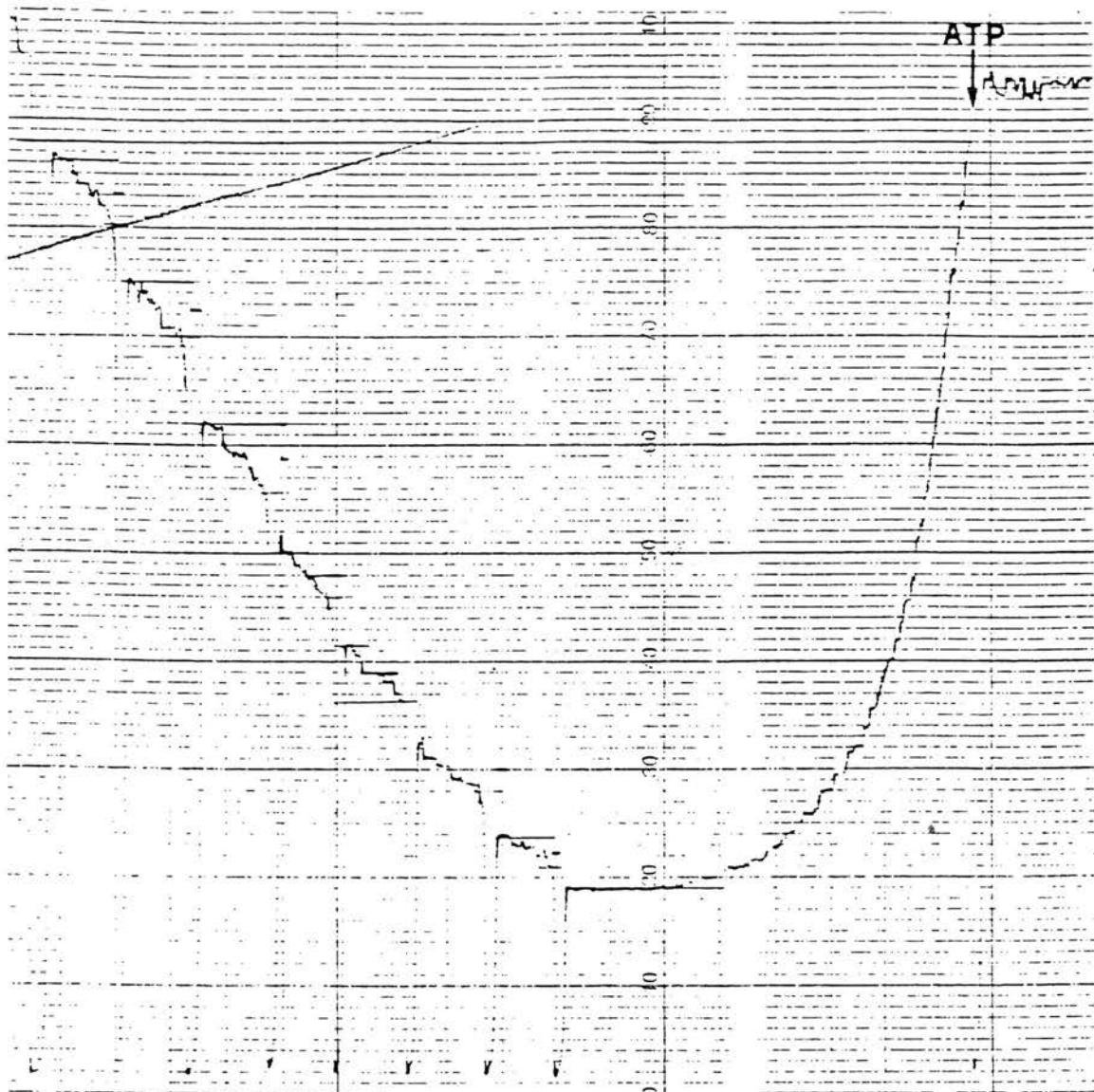


Fig. 3.17. Titration of 9-aminoacridine fluorescence quenching. Ten μl of reconstituted ATPase (about 5 μg) were incubated in assay medium (see Chapter 2, section 2.7). H^+ -translocation was activated by adding ATP, generating an inside-acid pH which led to quenching of 9-aminoacridine fluorescence. Once maximal quenching was achieved the external pH of the solution was changed by addition of 1 μl aliquots of 200 mM HCl. The extent of quenching was reduced by the decrease in the external pH.

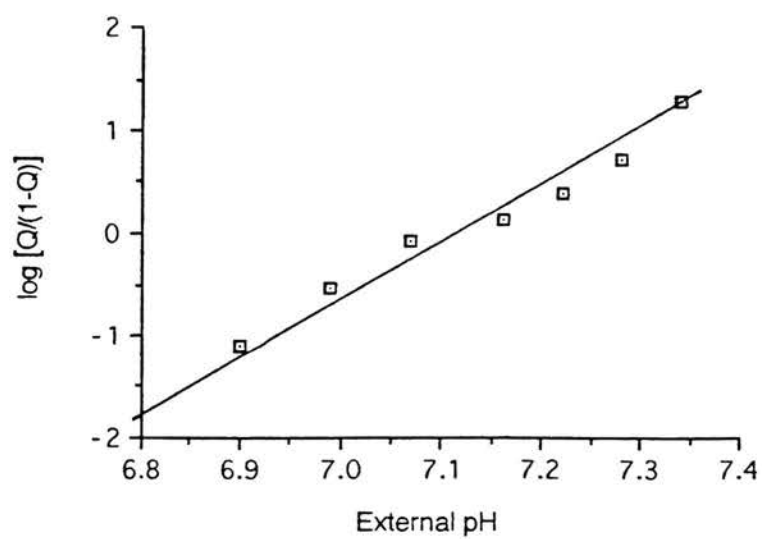


Fig. 3.18. Representation of external pH vs $\log [Q/(1-Q)]$ for 9-amino-acridine in the reconstituted ATPase.

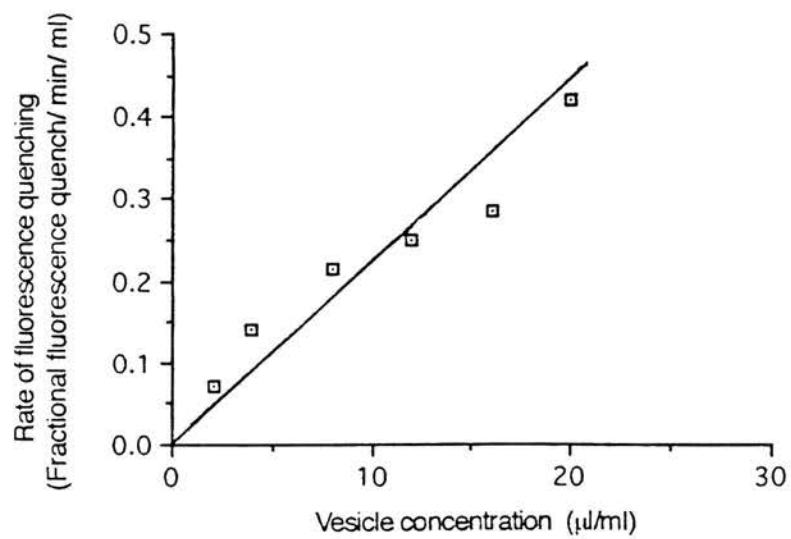


Fig. 3.19. Dependence of the initial rate of quenching of 9-aminoacridine fluorescence on vesicle concentration.

3.6. Discussion

The initial aim of the project was to develop a procedure for the purification and reconstitution of the *Kalanchoë* tonoplast ATPase. Two approaches were used in attempts to purify the ATPase; HPLC of the detergent solubilized ATPase complex and fractionation of tonoplast membranes with Triton X-114. Two non-ionic detergents C₁₂E₉ and MEGA-8 were assessed for their ability to solubilise the ATPase complex. The highest ATPase activity was achieved when tonoplast membranes were solubilised in 1.67% (w/v) MEGA-8. Attempted purification of the ATPase by HPLC of solubilised tonoplast membranes gave poor separation of the constituent membrane proteins suggesting that they had aggregated. To overcome this problem 100 mM KCl was added to the column buffer. However, HPLC of the solubilised tonoplast proteins under these modified conditions resulted in partial dissociation of the ATPase complex during purification. The dissociation of the ATPase complex may have been due to the presence of 100 mM KCl in the column buffer. Dissociation of V-type ATPases under conditions of high ionic strength has been widely reported in the literature (see Chapter 1, section 1.2.3.4 and references therein). The lack of success in purifying the ATPase complex by HPLC, led to this approach being abandoned. Fractionation of tonoplast membranes with Triton X-114 gave a product of specific activity comparable to that of other plant ATPases purified chromatographically. The purification and reconstitution protocol developed from the Triton X-114 procedure can be completed within 2 h and gives a product that is stable at 4°C.

For fractionation of the tonoplast membranes with Triton X-114, a detergent concentration of 6.2 mg/ml gave the best results (Fig. 3.10). The conditions used for solubilization of the ATPase in *n*-octyl glucoside were

critically important for successful reconstitution of the plant enzyme. When reconstituting the chromaffin granule V-type ATPase (Pérez-Castiñeira & Apps, 1990), the enzyme was solubilized in *n*-octyl glucoside before adding purified lipids. However, this method proved to be unsuitable when applied to the tonoplast ATPase, as this enzyme was rapidly inactivated when solubilized in *n*-octyl glucoside alone. In order to reconstitute the plant enzyme in an active state, it was essential that the solubilization with *n*-octyl glucoside was performed in the presence of lipids already dissolved in the detergent.

The tonoplast ATPase was reconstituted with a mixture of PC, PS and cholesterol in the ratio 27:53:20 (by weight), which was found to be optimal for both ATP-hydrolysis and H⁺-translocation. H⁺-translocation was much more sensitive to changes in the lipid composition of vesicles than was ATPase activity. This was illustrated by changes in the cholesterol concentration; although this lipid was critical for H⁺-translocation, its addition had little effect on the ATPase activity of the enzyme (Figs. 3.5 and 3.8).

The phospholipid composition of the native tonoplast membrane from *Kalanchoë daigremontiana* has been reported (Haschke *et al.*, 1990). This study revealed that free sterols were the major component of tonoplast membranes, accounting for 21.4% of the total lipids. Free sterols are structurally similar to cholesterol and probably enhance the mechanical stability of the tonoplast membrane in much the same way that cholesterol strengthens mammalian membranes.

The concentration of cholesterol in the lipid mixture used to reconstitute the ATPase is 20%, which is virtually identical to the concentration of free sterols in the tonoplast. Therefore a cholesterol/sterol concentration of approximately 20% is probably an essential pre-requisite for producing

sealed vesicles, suggesting that the lipid conditions necessary for H⁺-translocation probably reflect this requirement, rather than the intrinsic lipid dependence of the ATPase itself. It was important to establish this empirical requirement in order to carry out kinetic measurements on H⁺-translocation (Chapter 4).

Tonoplast preparations from *Kalanchoë daigremontiana*, from which purification of the vacuolar (V-type) ATPase is described in this chapter, are a particularly good source of the enzyme as judged by their high specific ATPase activity and the relative abundance of the ATPase subunits in the unfractionated membranes (Fig. 3.11). Electron microscopy of freeze-fractured tonoplast membranes from *K. daigremontiana* indicated that the V-type ATPase may comprise up to 36% of total tonoplast protein (Klink *et al.* 1990), an estimate consistent with the distribution of protein and ATPase activity in fractions following size exclusion chromatography of solubilized *Kalanchoë* tonoplast membranes (Bremberger *et al.*, 1988). The purified enzyme contains polypeptides of apparent molecular mass 72, 57, 48, 42, 39, 33 and 16 kDa, and these co-sediment during glycerol-gradient centrifugation. This subunit composition is very similar to that determined by Bremberger *et al.* (1988) for *K. daigremontiana*, except that our work has revealed an additional polypeptide of 39 kDa. Although a large (approx. 100 kDa) glycosylated subunit is a component of most, if not all animal V-type ATPases (Moriyama & Nelson, 1987; Apps *et al.*, 1989; Moriyama & Futai, 1990; Nelson, 1991; Gillespie *et al.*, 1991), the function of such a subunit remains unknown. We found no evidence for a subunit of this size in the *Kalanchoë* enzyme, either by gel staining or by immune blotting, although small amounts phosphoenolpyruvate carboxylase (one of the major leaf proteins in CAM plants) co-purified with the ATPase. However,

the presence of a 100 kDa subunit in the vacuolar H⁺-ATPase of red beet has been reported (Parry *et al.*, 1989), so it is still unclear whether a polypeptide of 100-120 kDa is an essential component of all V-type ATPase, or is a species- or tissue-specific subunit.

Reconstitution of the ATPase into sealed vesicles able to support a measurable transmembrane pH difference allows the use of fluorescent probes for assaying the activity of the enzyme. The validity of using the initial rate of quenching of 9-aminoacridine fluorescence as a measure of the H⁺-translocation activity of the enzyme was tested by titration of the quench produced by the reconstituted ATPase with HCl. This showed that the probe did not behave as predicted by Schuldiner equation, since a plot of $\log [Q/(1-Q)]$ (where Q is the extent of quenching) against external pH (pH_{out}) produced a straight line whose slope was greater than 1.0. However, the fact that 9-aminoacridine responded in a linear fashion to changes in pH_{out} and that the initial rate of fluorescence quenching was found to be directly proportional to the amount of protein present in the assay, indicated that the initial rate of quenching of 9-aminoacridine fluorescence was a true measurement of the rate of H⁺-translocation of the ATPase.

The generation of a proton concentration difference (as shown by fluorescence quenching) was absolutely dependent on ATP (Fig. 3.16a) and Mg²⁺ (Fig. 3.16b), providing convincing evidence that the reconstituted ATPase created this gradient. Valinomycin (in the presence of K⁺) was also essential in the formation of a proton gradient (Fig 3.16c). The presence of this ionophore prevents the generation (through electrogenic H⁺-translocation) of a transmembrane potential (inside positive), which would oppose the further entry of protons into the vesicles and would thus impede

the formation of a large proton gradient.

Addition of the ionophore nigericin dissipates the proton concentration difference across the membranes by catalysing the exchange of K^+ for H^+ (Nicholls, 1982), restoring the fluorescence to initial values (see Fig. 3.16a, b and c) demonstrating the link between the proton gradient and fluorescence quenching. On addition of nigericin the recorded fluorescence instantaneously returns to basal levels confirming that the response of the probe is not limiting in the measurement of ΔpH generation.

The Triton X-114 purification method provides a rapid and convenient method for preparing vesicles containing purified *Kalanchoë* tonoplast ATPase, which are able to support measurable H^+ -translocation. H^+ -translocation, the primary function of the enzyme, is readily measured by the use of fluorescent probes. This permits study of the properties of the tonoplast ATPase in more detail, particularly the kinetic and regulatory characteristics of this enzyme complex (see Chapter 4). So far these have only been investigated by measurements of ATP hydrolysis in tonoplast membranes. A procedure for the purification and reconstitution of the tonoplast H^+ -ATPase from mung bean has recently been reported (Kasamo *et al.*, 1991). Although the specific activity of this preparation was not reported, the rate of H^+ -translocation was apparently much less than for the reconstituted *Kalanchoë* ATPase; in terms of fractional fluorescence quenching the rates are 0.0005 and 0.148 per min per μg protein for the mung bean and *Kalanchoë* enzymes, respectively. Also, the method for the reconstitution of the mung bean ATPase involved glycerol gradient and column chromatography steps and was therefore considerably longer than the Triton X-114 procedure.

CHAPTER 4

4. Investigation of the kinetic and regulatory properties of the reconstituted tonoplast ATPase

4.1. Introduction

One of the most characteristic features of CAM plants is their nocturnal accumulation of large amounts of malic acid in the vacuoles of photosynthetic cells. This energy-requiring process is believed to be driven by the proton electrochemical gradient, which is created by the electrogenic, H⁺-translocating ATPase at the tonoplast. The magnitude of this driving-force is given by:

$$\Delta\mu_{H^+} = \Delta\Psi - \frac{2.3 RT}{F} \Delta pH \quad (1)$$

where F is the Faraday constant, R is the gas constant and T is the absolute temperature. $\Delta\Psi$ is the membrane potential in mV and ΔpH is the pH difference across the vacuole membrane (defined as $pH_{out} - pH_{in}$).

For *K. daigremontiana*, there has been a detailed assessment of the energetic feasibility of nocturnal malic acid accumulation as driven by an H⁺-pumping ATPase at the tonoplast (Lüttge *et al.*, 1981; Smith *et al.*, 1982). From these thermodynamic evaluations it was concluded that the ATPase functions with a stoichiometry of 1 ATP hydrolysed for 2H⁺ transported (see Chapter 1, section 1.1.3.1, for detailed discussion). A stoichiometry of 2H⁺/ATP has also been quoted for the vacuolar H⁺-ATPase from bovine chromaffin granule membranes (Flatmark & Ingebretsen, 1977; Johnson *et al.*, 1982).

Knowing the stoichiometry of the ATPase it is possible to calculate the

theoretical maximum pH gradient (ΔpH) which the enzyme can be generate across the vacuole membrane, by relating $\Delta\mu_{\text{H}^+}$ to the free energy of ATP hydrolysis in the cytoplasm (ΔG_{ATP}), assuming that conditions of thermodynamic equilibrium are achieved.

ΔG_{ATP} is the sum of the standard Gibbs free energy change of ATP hydrolysis, $\Delta G^{\circ}_{\text{ATP}}$, and the mass action ratio for ATP hydrolysis ($[\text{ADP}] [\text{P}_i] / [\text{ATP}]$) as follows:

$$\Delta G_{\text{ATP}} = \Delta G^{\circ}_{\text{ATP}} + 2.3 RT \log \left(\frac{[\text{ADP}] [\text{P}_i]}{[\text{ATP}]} \right) \quad (2)$$

In *Kalanchoë* species ΔG_{ATP} has been estimated to be about -54 kJ/ mol (Lüttge, 1987). ΔG_{ATP} is related to $\Delta\mu_{\text{H}^+}$ as follows:

$$\Delta G_{\text{ATP}} = - nF \Delta\mu_{\text{H}^+} \quad (3)$$

where n is the apparent number of protons pumped per ATP hydrolysed.

Taking $n=2$ and $\Delta G_{\text{ATP}} = -54$ kJ/ mol, we obtain a value for $\Delta\mu_{\text{H}^+}$ of 280 mV (equivalent to -27 kJ/ mol). Under conditions of thermodynamic equilibrium, if $\Delta\Psi = 0$ equation (1) can be simplified to:

$$\Delta\mu_{\text{H}^+} = - \frac{2.3 RT}{F} \Delta\text{pH} \quad (4)$$

Substitution of the value for $\Delta\mu_{\text{H}^+}$ of 280 mV into equation (4) predicts a maximal value for ΔpH of 4.7.

In reality, the tonoplast ATPase of *K. daigremontiana* generates a maximum ΔpH of 3.6 (Jochem *et al.*, 1984), indicating that the enzyme must be kinetically regulated. The argument can be extended to the various acidic compartments within eukaryotic cells, such as lysosomes, endosomes, Golgi stacks and secretory vesicles, in which the measured

differences in ΔpH again suggest regulation of the H^+ -ATPases, either directly or indirectly through the dissipation of $\Delta\Psi$ by anion-conducting channels (Barasch *et al.*, 1988). The acidification of sealed tonoplast vesicles has been shown to be dependent upon permeant anions, in particular chloride and malate (Jochem *et al.*, 1984; Jochem & Lüttge, 1987; White & Smith, 1989). In sealed vesicles these anions can exert an effect on the ATPase in one of two ways: either by direct stimulation of ATPase activity, or by dissipation of $\Delta\Psi$ established during electrogenic, ATP-driven proton transport through a parallel anion flux via anion channels. This uptake of anions would itself be driven by the $\Delta\Psi$ component of $\Delta\mu_{\text{H}^+}$.

The development of a protocol for the purification and reconstitution of the *Kalanchoë* vacuolar ATPase (see Chapter 3) permitted some basic kinetic experiments and investigation of the mechanism of action of the reconstituted enzyme under conditions in which anion channels should not be present. Previously, kinetic studies of the tonoplast ATPase had been limited to work with native membranes (Sze, 1985).

4.2. Methods

Purification and reconstitution of the tonoplast ATPase are described in Chapter 3, section 3.2.2. Measurement of ATP-hydrolytic and H^+ -translocation activities are described in Chapter 2, sections 2.6 and 2.7, respectively.

Initial-rate data were fitted to the appropriate rate equations (the Michaelis equation, the Hill equation or a rate equation describing allosteric inhibition) by non-linear regression analysis using a computer program written in C

programming language and run on the Sequent computer of Edinburgh University Computing Service under UNIX. The program produced optimized values for parameters in the respective functions, although in some cases (e.g. when a parameter was expected to be an integral number) the parameter was fixed. The program also yielded theoretical values of initial rates, calculated from the optimized parameters; these were used for graphical comparison of the experimental and calculated data points.

The computer program was kindly provided and run by Dr G.L. Atkins, Department of Biochemistry, University of Edinburgh.

4.3. Kinetic studies of the reconstituted tonoplast ATPase

4.3.1. Reproducibility of the 9-aminoacridine quenching assay

The error distribution in measurement of the initial rate of 9-aminoacridine quenching was assessed by carrying out ten replicate determinations for each of three different rates. Rates were produced by using the same enzyme concentration with different concentrations of MgATP. The data thus obtained were analysed. It was found that the variances were proportional to the square of the rates. The weighting function was obtained by weighting equally each of the variance values and applying least squares regression (see Table 4.1).

	<u>ATP concentration</u>		
	Low	Medium	High
Mean rate	6.94	27.2	146.1
Standard deviation (SD)	0.73	1.45	5.49
Variance (= SD ²)	0.53	2.10	30.1
Variance/ Mean	0.077	0.076	0.206
Mean ²	48.2	761.8	23145

Table 4.1. Error structure for the 9-aminoacridine quenching assay. Ten estimates were made with each of three ATP concentrations. From the data it was determined that: $\text{Variance} = 0.754 + 0.001377(\text{rate})^2$.

4.3.2. The dependence of the rate of H⁺-translocation on substrate concentration

The reconstituted ATPase vesicles undergo rapid, ATP-dependent acidification, as revealed by the quenching of 9-aminoacridine fluorescence. The dependence of this rate on the concentration of MgATP²⁻ was studied (in the presence of a fixed free Mg²⁺ concentration of 1 mM). Initial rates were fitted to the Michaelis-Menten equation using the method described in section 4.2 of this chapter. The data were found to fit a simple Michaelis function, with $K_m = 33 \pm 1 \mu\text{M}$ (Fig. 4.1a).

4.3.3. The dependence of the rate of ATP-hydrolysis on substrate concentration

The kinetics of ATP-hydrolysis were more complex than those for H⁺-translocation and showed deviation from Michaelis-Menten kinetics (Fig. 4.1b). The data for ATP-hydrolysis were fitted to three different functions: the sum of two Michaelis-Menten equations, the sum of three Michaelis-Menten equations, and the empirical Hill equation:

$$v_o = V_{\max} / (1 + K/[S]^{n_h}) \quad (5)$$

The data were fitted to all three functions by the method described in section 4.2. The best fit of these data was obtained with the double Michaelis-Menten equation, assuming two independent catalytic sites, with K_m values of 776 ± 63 and $1.9 \pm 1.2 \mu\text{M}$, and V_{\max} values of 3.65 and 0.27 $\mu\text{mol/mg/min}$, respectively. The large S.D. in the lower K_m value is due to the difficulty of measuring reaction rates at very low concentrations of MgATP.

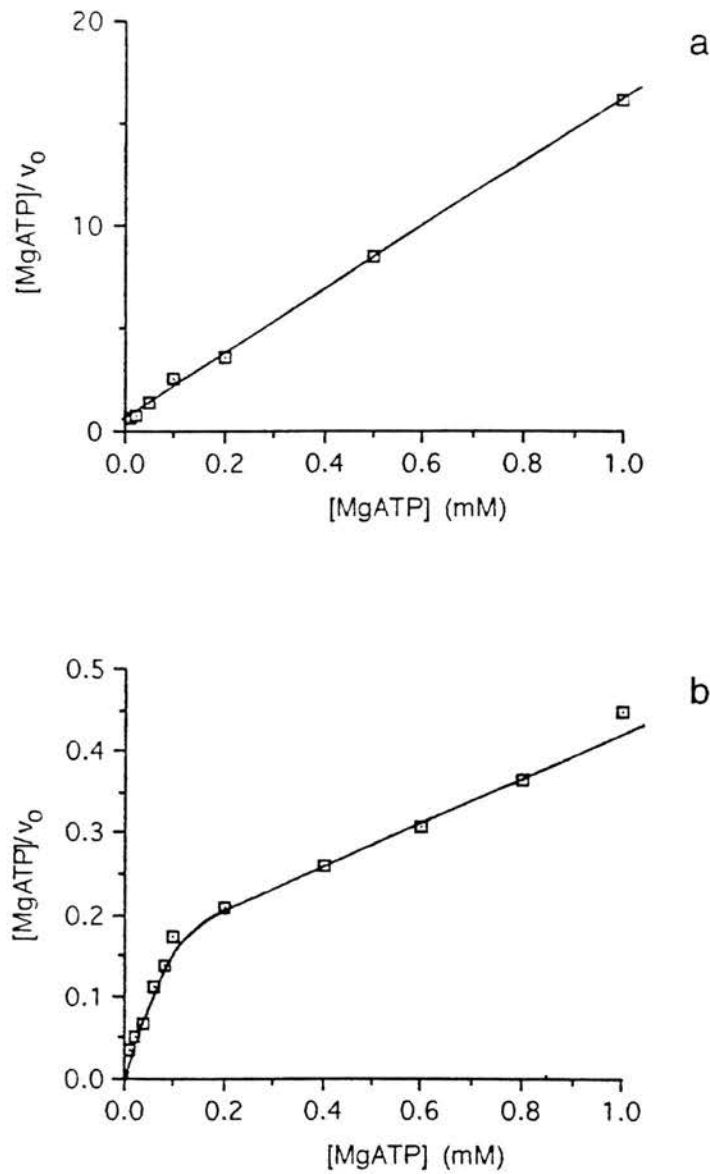


Fig. 4.1. Kinetics of the reconstituted ATPase. (a) Hanes plot of the dependence of the rate of H^+ -translocation on the concentration of MgATP. The data points are experimental (each a mean of 4 observations). The line is the best fit, calculated for $K_m = 33 \mu M$. Initial rates (v_0) are expressed as fractional fluorescence quench/ min per μg protein. (b) Hanes plot of the dependence of the rate of ATP hydrolysis on the concentration of MgATP. The data points are experimental (each a mean of 2 observations). The line is the best fit, calculated for K_m values of 776 and $1.9 \mu M$. Initial rates (v_0) are expressed as μmol ATP hydrolysed/ min/ mg protein.

Previous studies of ATPase activity in fractions of the native tonoplast from *Kalanchoë daigremontiana* yielded apparent K_m values of 310 μM (Smith *et al.*, 1984a) to 810 μM (Jochem & Lüttge, 1987), depending on the ionic composition of the assay medium.

4.3.4. Substrate specificity of the reconstituted ATPase

The substrate specificity of the reconstituted ATPase was investigated by measuring initial rates of H^+ -translocation produced by a range of different nucleoside triphosphates. The kinetic parameters are shown in Table 4.2. In terms of the relative V_{max} values obtained with the different nucleoside triphosphates (as their magnesium complexes), the relative substrate effectiveness is

$$\text{dATP} > \text{ATP} > \text{ITP} > \text{GTP} = \text{UTP} \gg \text{CTP}$$

With CTP as substrate the activity of the ATPase was so low that the K_m value could not be measured. This result demonstrates that hydrolysis of nucleoside triphosphates other than ATP, which was observed previously in preparations of isolated vacuoles (Smith *et al.*, 1984a), is a genuine property of the ATPase.

The true substrate of the ATPase is not free ATP, but a complex of ATP and a divalent cation (e.g. MgATP^{2-}). Table 4.2 shows the effect of different divalent cations on the H^+ -translocating ability of the reconstituted ATPase when they are substituted for Mg^{2+} . As found with other vacuolar ATPases, Ca^{2+} can replace Mg^{2+} ; and MnATP^{2-} is actually a better substrate than MgATP^{2-} (O'Neill *et al.*, 1983; Flatmark *et al.*, 1985). No H^+ -translocation was measurable with Zn^{2+} as the activatory cation; investigation of the

Substrate	K_m (μM)	Relative V_{max} (%)
MgATP	33 ± 1	100
MgdATP	17 ± 1	204
MgCTP	n.d.	4
MgGTP	86 ± 4	56
MgITP	162 ± 5	70
MgUTP	263 ± 9	46
CaATP	24 ± 1	44
MnATP	10 ± 0.3	111

Table 4.2. Kinetic parameters of the reconstituted ATPase. Initial rates of H^+ -translocation were determined by the quenching of 9-aminoacridine fluorescence, and fitted to the Michaelis equation. Because the rates were measured in arbitrary units, the quoted values of V_{max} are relative to those with MgATP. Each data set contained 28 rate measurements, encompassing a 100-fold range of substrate concentration. n.d., not determined.

effects of Co^{2+} or Ni^{2+} was precluded by their quenching of the fluorescence of 9-aminoacridine.

4.3.5. Inhibition of the reconstituted ATPase by nitrate and bafilomycin

Nitrate is a characteristic inhibitor of V-type ATPases (Sze, 1985; Forgac, 1989). The inhibition by nitrate of ATP hydrolysis and H^+ -translocation is shown in Fig. 4.2. Nitrate is a noncompetitive inhibitor of H^+ -translocation, the K_m for MgATP^{2-} being unaltered by concentrations of NO_3^- up to 50 mM (Fig. 4.3). The Cl^- -dependent H^+ -translocation activity of the tonoplast H^+ -ATPase from oat roots is also noncompetitively inhibited by nitrate (Churchill & Sze, 1984). As with native membranes from *Kalanchoë* (Jochem* & Lüttge, 1987), H^+ -translocation is more sensitive to nitrate inhibition than is ATP hydrolysis, the inhibitor concentrations that produce half-maximal inhibition of the reconstituted enzyme being 4 and 9 mM, respectively.

A more specific and potent inhibitor of V-type ATPases is bafilomycin A_1 (Bowman *et al.*, 1988c), which inhibits ATP hydrolysis and H^+ -translocation in parallel (Fig. 4.4). The apparent I_{50} value in this experiment is 0.18 ng bafilomycin/ μg protein which would extrapolate to saturation at approximately 0.36 ng bafilomycin/ μg protein. This can be recalculated as 0.3 mol inhibitor/mol ATPase (assuming that the enzyme is pure and has a molecular mass of 500 kDa). A comparable result has been obtained with the purified vacuolar ATPase from oat roots, where it was estimated that about 0.7 mol bafilomycin/mol ATPase was required to achieve 84%

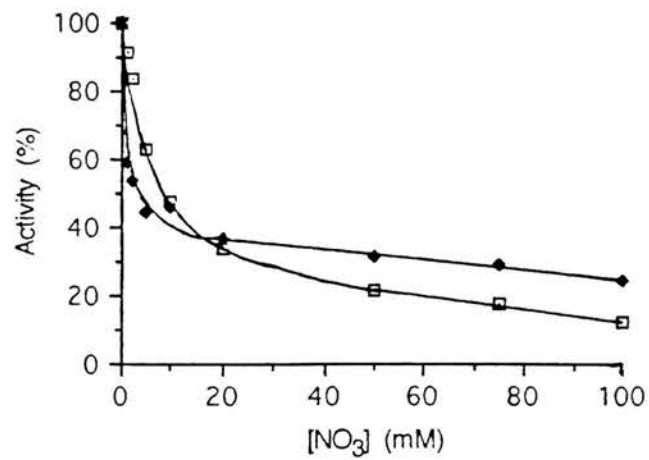


Fig. 4.2. Inhibition of the reconstituted ATPase by nitrate.

(♦) H⁺-translocation; (◻) ATP hydrolysis.

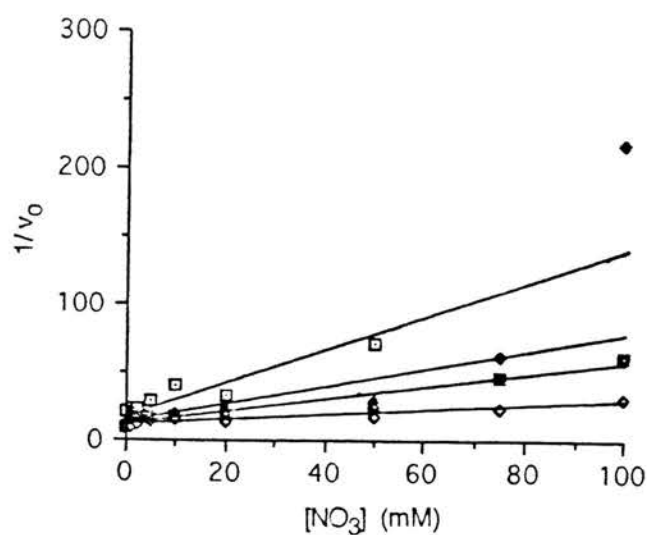


Fig. 4.3. Effect of nitrate on the H⁺-translocating activity of the reconstituted ATPase. Dixon plot of the inhibition of H⁺-translocation with nitrate in the presence of: 10 μM (□), 50 μM (♦), 100 μM (■), and 500 μM (○) ATP respectively. Initial rates (v₀) are expressed as fractional fluorescence quench/ min per μg protein.

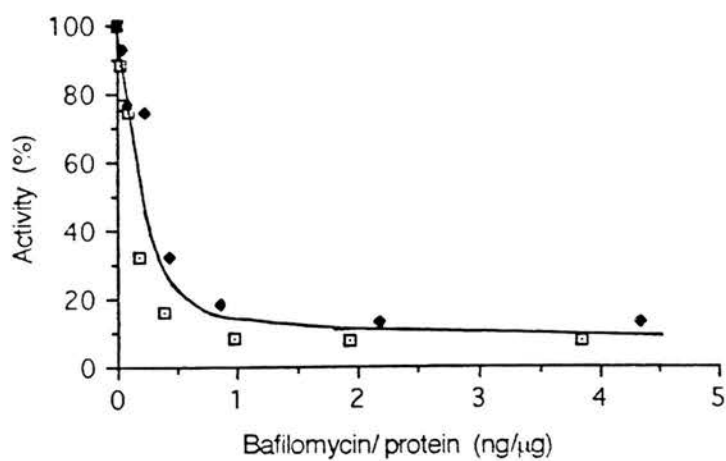


Fig. 4.4. Inhibition of the reconstituted ATPase by bafilomycin A₁.

(◆) H⁺-translocation; (◻) ATP hydrolysis.

inhibition (Ward & Sze, 1992). Bowman *et al* (1988c) estimated that an inhibitor concentration of 1 mol bafilomycin/mol of 70 kDa polypeptide (or 3 mol bafilomycin/ATPase holoenzyme) was required for 50 % inhibition of the *Neurospora* ATPase. The results obtained with the reconstituted *Kalanchoë* ATPase and the purified ATPase from oat roots support the idea that bafilomycin reacts stoichiometrically with V-type ATPases (Bowman *et al.*, 1988c) . However, even at high concentrations, bafilomycin does not totally inhibit the *Kalanchoë* enzyme. There is a residual activity (approximately 10% of the uninhibited rate) which is resistant to this inhibitor. This activity was found to be nitrate-sensitive and to display the complex kinetics of ATP hydrolysis described above (Fig. 4.5).

4.3.6. Inhibition of the reconstituted ATPase by nucleoside diphosphates

Five nucleoside diphosphates, ADP, CDP, GDP, IDP and UDP, were tested for their ability to inhibit the reconstituted ATPase. ADP and GDP inhibit H⁺-translocation most strongly, while IDP is only mildly inhibitory. No significant inhibition was observed with either CDP or UDP (Fig 4.6a). The kinetic parameters are shown in Table 4.3. Fitting of the data to the Hill function reveals that in the absence of inhibitors $n_h < 1$, but is significantly increased in the presence of ADP, GDP and IDP. This suggests that these nucleoside diphosphates induce positive cooperativity for substrate binding.

Of the five nucleoside diphosphates tested, ADP was found to inhibit the tonoplast ATPase most strongly. Therefore the inhibitory effects of this nucleoside diphosphate on the reconstituted enzyme were studied more closely. The inhibition of the H⁺-translocation activity of the reconstituted

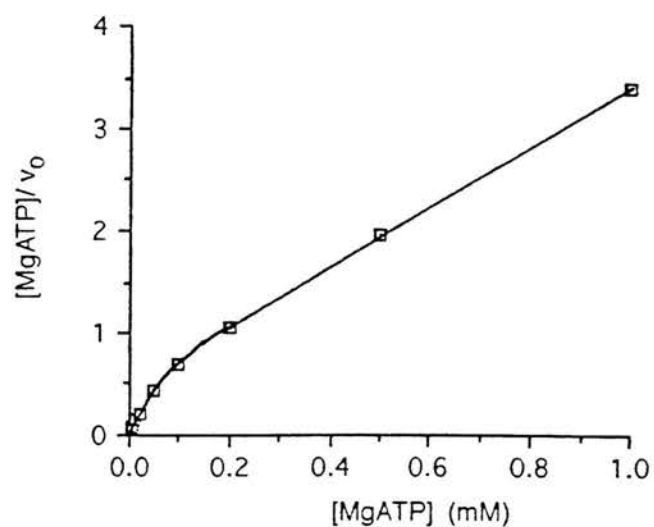


Fig. 4.5. Kinetics of bafilomycin resistant ATPase activity. Hanes plot of the dependence of the rate of ATP hydrolysis on the concentration of MgATP in the presence of 1 ng bafilomycin/ μg protein. The data points are experimental (each a mean of 3 observations). Initial rates (v_0) are expressed as μmol ATP hydrolysed/ min/ mg protein.

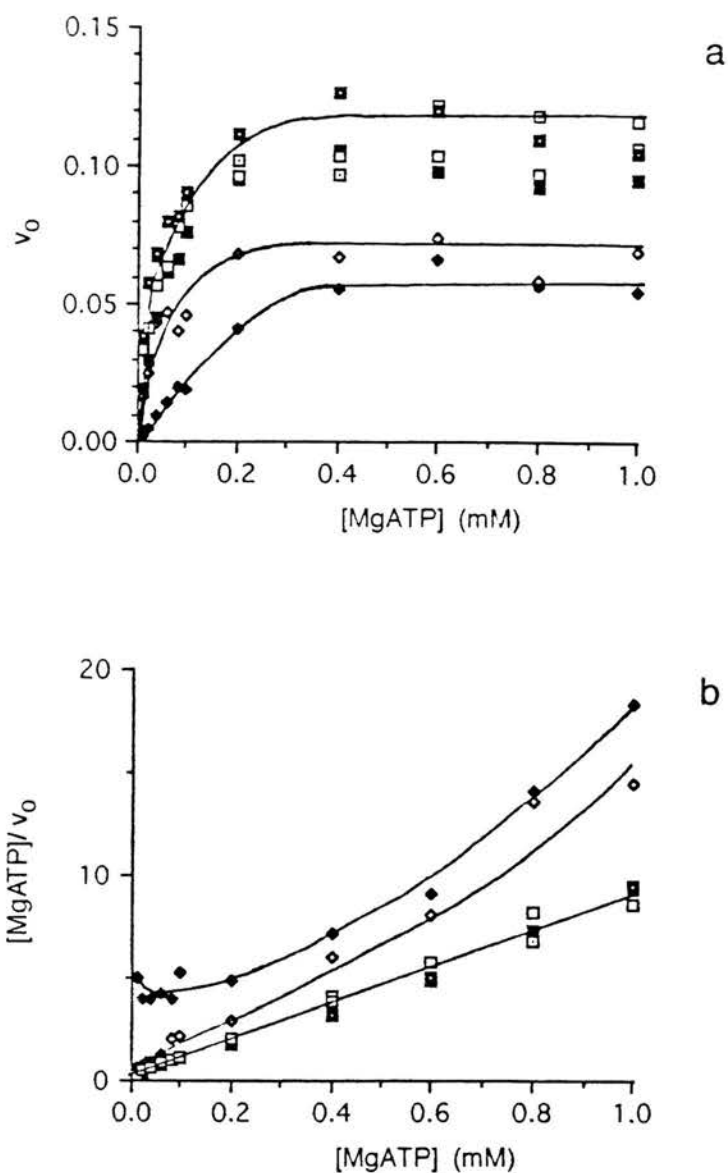


Fig. 4.6. Inhibition of the reconstituted ATPase by different nucleoside diphosphates. MgATP concentration dependence of the rate of H^+ -translocation of the reconstituted ATPase in the absence of inhibitor (\square) and in the presence of 200 μ M of the following NDP's: ADP (\blacklozenge), CDP (\blacksquare), GDP (\blacklozenge), IDP (\blacksquare), and UDP (\square). (a) Michaelis plot; (b) Hanes plot. Initial rates (v_0) are expressed as fractional fluorescence quench/ min per μ g protein.

Inhibitor	V	K (μ M)	n_h
None	$0.122 \pm 3.30 \times 10^{-3}$	28.2 ± 2.6	0.72 ± 0.05
200 μ M ADP	$0.071 \pm 1.62 \times 10^{-3}$	136.9 ± 8.2	1.58 ± 0.10
200 μ M CDP	$0.122 \pm 3.02 \times 10^{-3}$	26.3 ± 2.1	0.82 ± 0.006
200 μ M GDP	$0.072 \pm 2.38 \times 10^{-3}$	37.4 ± 3.9	0.93 ± 0.09
200 μ M IDP	$0.102 \pm 1.54 \times 10^{-3}$	42.9 ± 1.8	1.17 ± 0.05
200 μ M UDP	$0.117 \pm 3.02 \times 10^{-3}$	38.8 ± 3.3	0.78 ± 0.05

Table 4.3. Effect of nucleoside diphosphates on the kinetic parameters of the reconstituted ATPase. Initial rates of H^+ -translocation were determined by the quenching of 9-aminoacridine fluorescence, and fitted to the Hill equation. The inhibitory effects of each nucleoside diphosphate were measured over a 100-fold range of substrate concentration. Rates of H^+ -translocation (V) are expressed as fractional fluorescence quench/ min per μ g protein.

Kalanchoë enzyme with ADP, over a range of inhibitor concentrations, is shown in Fig. 4.7a. The tonoplast ATPase demonstrated an increasingly sigmoidal dependence on MgATP^{2-} in the presence of progressively higher inhibitor concentrations, suggesting that ADP acts as an allosteric inhibitor inducing cooperative ATP binding at low substrate concentrations. However, fitting of this data to the Hill function gives $n_h > 1$ in the absence of ADP and this value does not increase greatly when the ADP concentration increases (see Table 4.4).

The complex nature of the ADP inhibition of the reconstituted ATPase can be seen in a Hanes plot of the inhibition data (Fig 4.7b). Of the other nucleoside diphosphates tested, only GDP displayed a similar pattern of inhibition (Fig 4.6b). This complex pattern of ADP inhibition has also been observed for the ATP-hydrolytic activity of the tonoplast ATPase from red beet (Bennett *et al.*, 1985).

4.4. Construction of a mathematical model for the regulatory properties of the *Kalanchoë* tonoplast ATPase

A major goal of the research was to characterise the structure and function of the *Kalanchoë* tonoplast ATPase. It was therefore important to construct a theoretical model for the enzyme that could quantitatively explain the results obtained in the regulatory studies. The “symmetry” (or “concerted transition”) model of allosteric regulation proposed by Monod *et al.* (1965) (often termed the MWC model) was used as the basis from which to approach the problem. This relatively simple model explains cooperative interactions in terms of molecular symmetry. Equations derived from this model contain a structural number n , distinct from the parameter n_h in the empirical Hill equation, which has no structural significance.

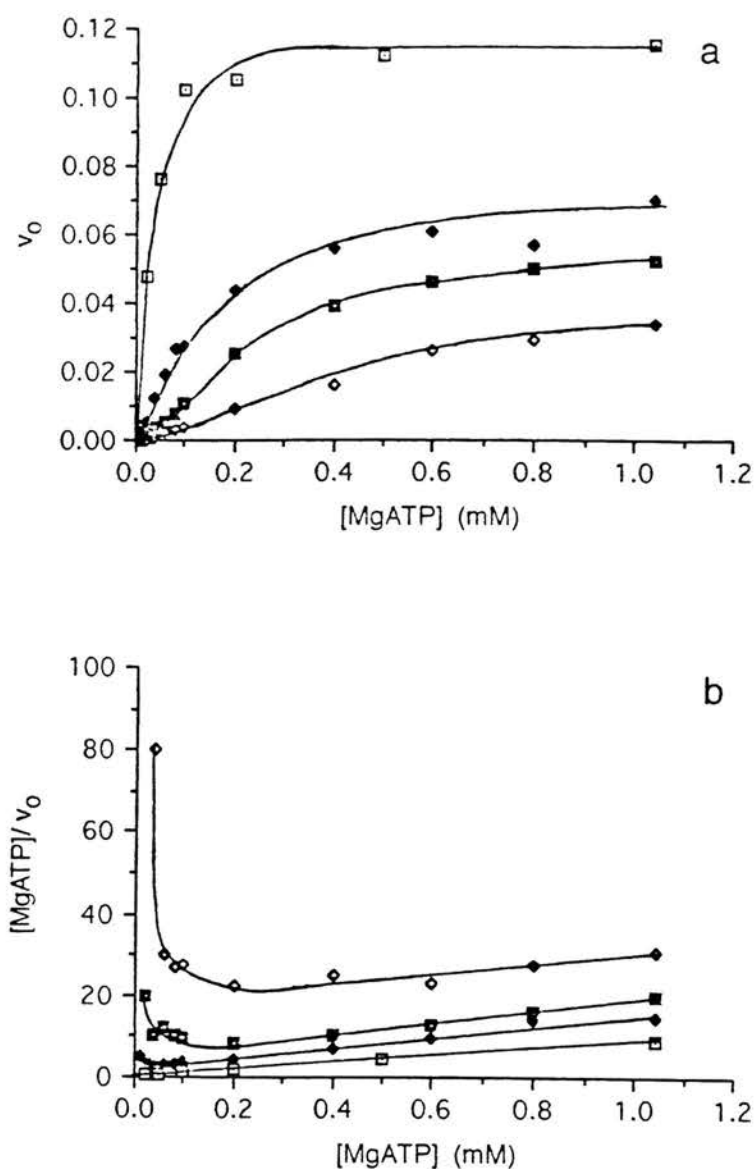


Fig. 4.7. Inhibition of the reconstituted ATPase by ADP. MgATP concentration dependence of the rate of H^+ -translocation of the reconstituted ATPase in the presence of: 0 μM (\square), 50 μM (\blacklozenge), 200 μM (\blacksquare), and 500 μM (\diamond) ADP respectively. (a) Michaelis plot; (b) Hanes plot. Initial rates (v_0) are expressed as fractional fluorescence quench/ min per μg protein.

Source	[ADP] (μM)	V	K (μM)	n_h
<i>Kalanchoë daigremontiana</i>	0	$0.116 \pm 3.0 \times 10^{-3}$	27 ± 2	1.26 ± 0.14
	20	$0.077 \pm 1.0 \times 10^{-3}$	98 ± 3	1.34 ± 0.05
	50	$0.068 \pm 1.4 \times 10^{-3}$	126 ± 6	1.31 ± 0.06
	100	$0.055 \pm 3.4 \times 10^{-3}$	166 ± 25	1.30 ± 0.15
	200	$0.057 \pm 0.4 \times 10^{-3}$	244 ± 3	1.60 ± 0.02
	500	$0.053 \pm 2.4 \times 10^{-3}$	667 ± 51	1.37 ± 0.05
Chromaffin granules	0	1.338 ± 0.031	26.6 ± 2.1	0.80 ± 0.02
	50	1.043 ± 0.053	165 ± 12.3	1.92 ± 0.13
	100	1.057 ± 0.045	224 ± 13.6	2.31 ± 0.12
	200	0.814 ± 0.042	315 ± 19.6	2.68 ± 0.17
	400	0.900 ± 0.163	627 ± 112	2.26 ± 0.30

Table 4.4. Effect of ADP on the kinetic parameters of the reconstituted tonoplast ATPase. Initial rates of H^+ -translocation were determined by the quenching of 9-aminoacridine fluorescence, and fitted to the Hill equation. The inhibitory effects of ADP were measured over a 100-fold range of substrate concentration. Rates of H^+ -translocation (V) are expressed as fractional fluorescence quench/ min per μg protein. For comparison, the kinetic parameters of the chromaffin granule ATPase are also shown. For this enzyme, the initial rates of H^+ -translocation were determined by the quenching of ACMA fluorescence, and fitted to the Hill equation. The inhibitory effects of ADP were measured over a 1000-fold range of substrate concentration. The data for the reconstituted chromaffin granule ATPase were kindly provided by Dr. J.R. Pérez-Castiñeira.

This is important information, since n indicates the number of functional subunits in the enzyme complex.

The MWC model postulates that enzymes which exhibit cooperative (positive homotropic) effects are composed of a number of identical subunits or protomers. A protomer is defined as a structure that is repeated several times within the enzyme complex, but is not necessarily a single polypeptide chain. In the simplest case, the enzyme exists as an equilibrium mixture of two states, known as R (relaxed) and T (taut), which differ in their binding and/or catalytic properties. Preferential binding of a ligand (either substrate or inhibitor) to one of the states displaces the conformational equilibrium towards that form of the enzyme, resulting in a change in the affinity of catalytic activity of the system as a whole. For this reason the system is compatible with cooperative binding of substrates or inhibitors (positive homotropic effects) but not with negative cooperativity.

An expression for the saturation function (Y_S), defined as [filled sites]/[total sites], can be derived from the model:

$$Y_S = \frac{(S/K_R) [1 + (S/K_R)]^{n-1} + L' (S/K_T) [1 + (S/K_T)]^{n-1}}{[1 + (S/K_R)]^n + L' [1 + (S/K_T)]^n} \quad (6)$$

where

$$L' = L(1 + I/K_I)^n$$

S is the substrate concentration, K_R is a dissociation constant of the substrate from the R state, L is the allosteric constant (the equilibrium constant for the structural transition, equal to $[T]/[R]$), I is the inhibitor concentration, K_I is the dissociation constant of the inhibitor from the T state, K_T is the dissociation constant of the substrate from the T state and n is the number of subunits of the complex. This expression is deduced with the assumption that the binding of the inhibitor to the R state is negligible.

The MWC model incorporates two extreme cases: "K-systems", in which

the substrate has different affinities for the T and R states but the catalytic activities of the two states are equal and “V-systems”, in which the substrate has the same affinity for the two states, which however differ in their catalytic activity. In either case, allosteric inhibitors (“negative homotropic effectors”) bind more tightly to the T than to the R state, while allosteric activators (“positive homotropic effectors”) bind more tightly to the R than to the T state. In K-systems, the presence of the inhibitor affects the apparent affinity of the substrate for the enzyme, i.e. changes the S_{50} value (the substrate concentration required to produce half maximal velocity), but does not affect the V_{\max} of the reaction. In V-systems, the presence of the inhibitor affects the activity of the enzyme but not its affinity for the substrate i.e. the V_{\max} is altered but not the value of S_{50} .

Both the S_{50} and V_{\max} for the tonoplast H^+ -ATPase are affected by the presence of inhibitors (see section 4.3.6). This means the ATPase displays a mixture of the properties of K-system and V-system enzymes, which can be explained by postulating that the protein has two states that differ both in their affinities for the substrate and their catalytic activities (Tipton, 1979). If we assume the saturation function (Y_S) is proportional to v_o/V_{\max} then from equation (6) the rate equation is:

$$\frac{v_o}{V_{\max}} = \frac{(S/K_R) [1 + (S/K_R)]^{n-1} + L' (S/K_T) [1 + (S/K_T)]^{n-1}}{[1 + (S/K_R)]^n + L' [1 + (S/K_T)]^n} \quad (7)$$

This expression can be simplified by assuming that the R-state is active and the T-state is inactive:

$$\frac{v_o}{V_{\max}} = \frac{(S/K_R) [1 + (S/K_R)]^{n-1}}{[1 + (S/K_R)]^n + L' [1 + (S/K_T)]^n} \quad (8)$$

The H⁺-translocation activity of the ATPase displays simple Michaelis-Menten kinetics in the absence of inhibitors. This suggests that, under these conditions, the R/T equilibrium is overwhelmingly toward the R-state (i.e. L is small). Thus in the absence of inhibitors, the T-state does not exert any effect on the saturation with substrate, and the enzyme behaves according to the Michaelis-Menten equation:

$$\frac{v_o}{V_{\max}} = \frac{(S/K_R) [1 + (S/K_R)]^{n-1}}{[1 + (S/K_R)]^n + L' [1 + (S/K_T)]^n} = \frac{S/K_R}{1 + S/K_R} = \frac{S}{K_R + S} \quad (9)$$

The cooperative effects which are observed, even at quite low ADP concentrations, can be explained if it is assumed that ADP has a very high affinity for the T-state (i.e. that K_i , the dissociation constant of ADP from the T-state, is small), thus displacing the allosteric equilibrium towards the inactive T-state and increasing the value of the apparent allosteric constant (L').

The discussion above leads us to the following qualitative predictions about the reconstituted *Kalanchoë* ATPase: K_R has a similar value to the K values obtained for MgATP in the absence of inhibitors, fitting the Hill equation (approx. 30 μ M); K_T is much larger than K_R so that the allosteric equilibrium cannot be displaced towards the T-state by binding of the substrate alone; L is small so that in the absence of negative effectors, the enzyme is mainly in the R-state; K_i for ADP is also small since relatively low concentrations of ADP are inhibitory; V_{\max} must be consistent with the experimental values and n is probably 2 or 3 according to the expected structure of the catalytic complex.

The variance in measurement of the initial rate of 9-aminoacridine quenching was found to be proportional to the square of the rates (see

section 4.3.1). Therefore the experimental data were fitted to the MWC model using the weighting function obtained for the 9-aminoacridine quenching assay.

The experimental data were fitted to expression (7). All parameters were allowed to "float" when the initial calculations were made and it was found that the values calculated for n were consistently close to 2 or 3; optimised values for the other parameters were then determined with n fixed at 2 or 3. The values obtained are shown in Table 4.5. Optimised theoretical data sets were also produced by the programme. The main observations when $n=2$ and when $n=3$ are that V_{\max} is underestimated compared to the experimentally derived value of 0.116 fractional fluorescence quench/ min per μg protein (see Table 4.4, line 1), and that K_i is larger than might be predicted. Also when $n=3$, the value optimized of L is 21.3 indicating that the enzyme is predominantly in the T-state even in the absence of inhibitors and effectors. The main problem is that there is a large standard error associated with this value for L . This problem is even worse when $n=2$ since the standard error is ten times bigger than the estimated value of L .

However, in equation (7) it has been assumed that there are equal numbers of binding sites for substrate and inhibitor, i.e. 2 or 3. Although symmetry might suggest that this is so, it is not necessarily the case. For a scheme in which the number of binding sites for the substrate is different to that for the inhibitor, a new parameter representing the number of inhibitor binding sites (m), which may not be equal to n , has to be introduced into the expression for the apparent allosteric constant:

$$L' = L [1 + (I/K_i)]^m \quad (10)$$

The experimental data were fitted to the new expression using two approaches: (1) n and m were assigned fixed integral values while the other

	K_R (μM)	V_{max}	L	K_T (μM)	K_I (μM)	Sum of squares of residuals
$n=2 \quad m=1$	3.2 ± 1.3	$0.086 \pm 6 \times 10^{-4}$	21.5 ± 18.3	293 ± 3	1.0 ± 0.1	4.8×10^2
$n=2 \quad m=2$	0.3 ± 1.5	$0.067 \pm 6 \times 10^{-4}$	3938 ± 47466	8525 ± 3053	8.7 ± 0.6	1.3×10^3
$n=3 \quad m=1$	12.1 ± 1.4	$0.088 \pm 6 \times 10^{-4}$	1.1 ± 0.9	142 ± 3	0.2 ± 0.1	5.1×10^2
$n=3 \quad m=2$	1.9 ± 1.4	$0.065 \pm 6 \times 10^{-4}$	557 ± 1270	593 ± 26	3.7 ± 0.3	1.2×10^3
$n=3 \quad m=3$	7.9 ± 1.7	$0.062 \pm 6 \times 10^{-4}$	21.3 ± 14.3	2729 ± 420	16.4 ± 1.1	1.6×10^3
$m=1^*$	0.5 ± 2.3	$0.086 \pm 6 \times 10^{-4}$	215 ± 1515	565 ± 72	1.5 ± 0.3	4.7×10^2

Table 4.5. Values of different parameters calculated by fitting experimental data to the equation of the allosteric model.
 See text for details. * n allowed to "float", calculated value $n=1.58$. V_{max} is measured in units of fractional fluorescence quench/ min per μg protein.
 From rate measurements in the absence of ADP V_{max} is estimated graphically to be 0.116 fractional fluorescence quench/ min per μg protein.

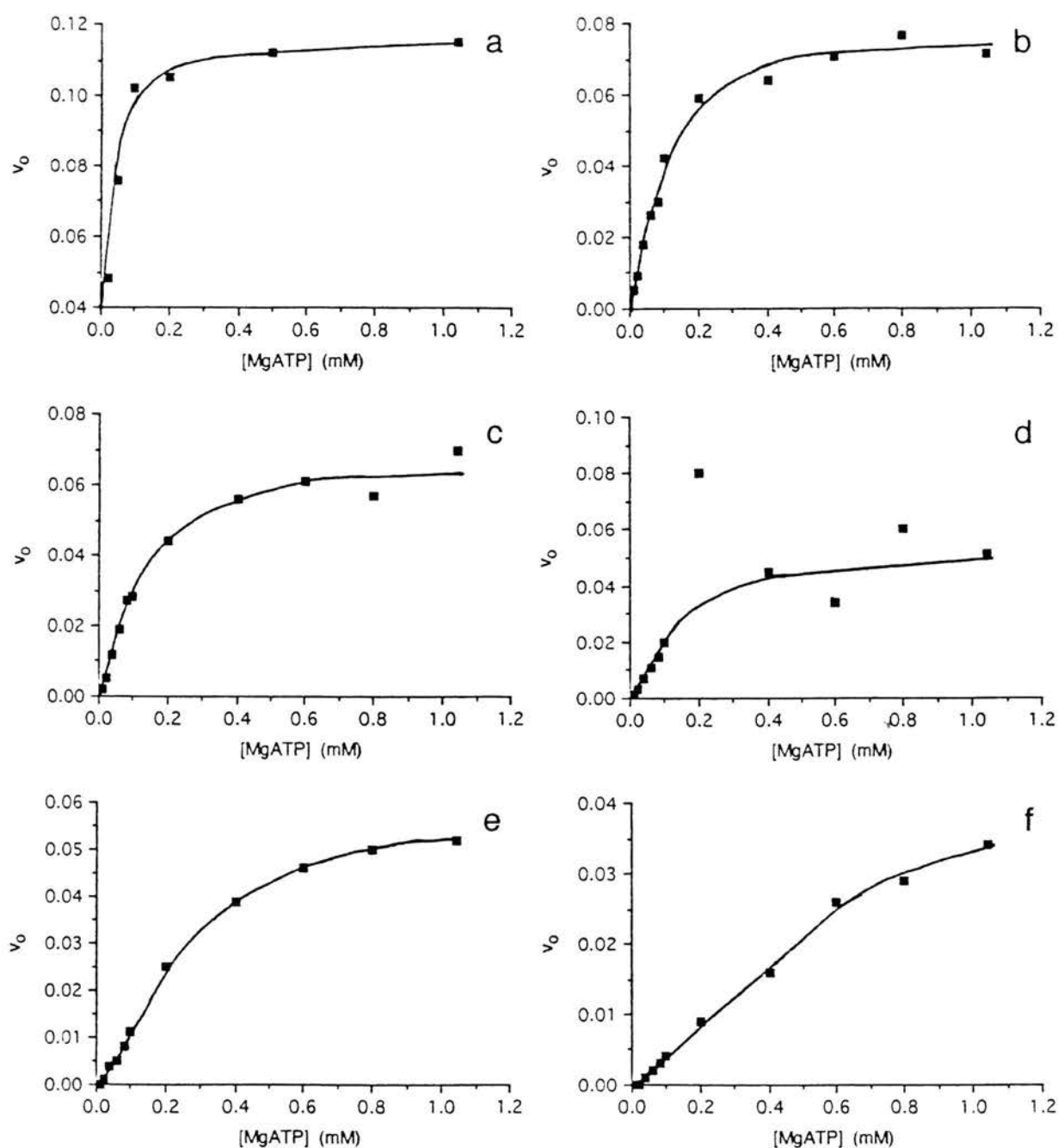


Fig. 4.8. Fitting of experimental data points to the allosteric equation.

Individual representations of the variation of the initial rate of 9-amino-acridine quenching with increasing amounts of MgATP in the presence of (a) 0, (b) 20, (c) 50, (d) 100, (e) 200 and (f) 500 μM ADP. Filled squares represent experimental data points and lines were calculated from the equations of the allosteric model (see text for details). Initial rates (v_0) are expressed as fractional fluorescence quench/ min per μg protein.

parameters were allowed to float; (2) m was fixed and all the other parameters, including n , were allowed to float. The results obtained are shown in Table 4.5. Although a good fit was obtained when m was fixed at 1 some of the values for the other parameters were difficult to accept: n was not close to an integral value (1.58), L was large and V_{\max} was again underestimated compared to the experimental value. For $n=2$ and $m=1$ the fitting was also very good though again, L was large and V_{\max} was underestimated. The fitting for $n=3$ and $m=1$ was only slightly worse than the latter case and as before V_{\max} was underestimated. When $n=3$ and $m=2$, the fitting was still reasonable, however, L was unacceptably large and as with all previous cases V_{\max} was underestimated.

The most realistic fittings were $n=3$, $m=1$ and $n=2$, $m=1$, although in the latter case the standard error obtained for value of L was almost as large as that estimated for the parameter itself. We can see from Fig 4.8 that there is a good fit to the experimental values suggesting that the behaviour of the enzyme is reasonably well explained by the model.

4.5. Discussion

The kinetic and regulatory properties of the *Kalanchoë* tonoplast ATPase were studied by measuring the initial rate of quenching of 9-aminoacridine fluorescence. The initial rate of fluorescence quenching was taken to be directly proportional to the rate of H^+ -translocation; an assumption that has been justified on theoretical grounds (Bennett & Spanswick, 1983) - see also Chapter 3, section 3.5.2. In the absence of any inhibitors and the presence of 1 mM free Mg^{2+} , the dependence of the rate of H^+ -translocation on the concentration of $MgATP^{2-}$ appeared to obey the

Michaelis equation, in that a Hanes plot was linear (Fig. 4.1a); the K_m value derived from this plot was 33 μM . Similar kinetics were observed with other purine nucleoside triphosphates or when Mg^{2+} was replaced with other cations (Table 4.2).

The substrate-specificity studies showed that only purine nucleoside triphosphates (ATP, GTP, ITP) can be considered as substrates for the enzyme, and that of the divalent cations tested only Mn^{2+} and Ca^{2+} could act as co-substrates substituting for Mg^{2+} . Indeed, MnATP was a better substrate than MgATP, with a lower K_m and similar V_{max} .

The kinetics of ATP hydrolysis were complex and produced non-linear Hanes plots (Fig. 4.1b). In principle plots of this type would be expected if two or more independent catalytic sites, with different K_m values, contribute to the measured rate; or alternatively if there is negative cooperativity between nucleotide-binding sites. The data shown in Fig. 4.1b could be satisfactorily fitted by summing two Michaelis functions, but were not better fitted by the sum of three Michaelis functions. Thus the experimental data supports the existence of two but not three different K_m values for ATP hydrolysis. Alternatively, the data could be fitted to the empirical Hill function (equation 5), with $n_h = 0.55$. This low value of the Hill coefficient would suggest negative cooperativity between ATP-binding sites, as occurs in F-type ATPases (Jault *et al.* 1991), but the fit was significantly worse than with the two- K_m model. It seems unlikely that these effects could be due to the reconstituted enzyme preparation being contaminated with other ATPases, but it might contain small amounts of a modified or dissociated form of the vacuolar ATPase, that has a lower K_m for MgATP but is incompetent in H^+ -translocation. Although these possibilities cannot be ruled out, it is important to note that similar kinetic behaviour has been reported with V-type ATPases

from other sources utilising different purification procedures and different activity assays. Hanada *et al.* (1990) determined three separate K_m values for the chromaffin granule enzyme, while Arai *et al.* (1989) found only two K_m values for the clathrin-coated vesicle ATPase, although in the latter case MgATP concentrations below 25 μ M were not tested. However, serious criticism can be made of the work reported by Hanada *et al.* in that their study was not exhaustive enough; firstly, the number of data points obtained was extremely small and secondly, the fitting of the data to double Michaelis-Menten kinetics used the statistically unsatisfactory Lineweaver-Burk plot, and was apparently done by eye.

Kinetic studies of F-type ATPases have revealed that these enzymes also have several values for K_m and V_{max} (see Table 4.6). For both V-type and F-type ATPases, increasing the MgATP concentration produces an increase in the apparent K_m and an increase in the rate of hydrolysis. This means that the binding of substrate appears to decrease the affinity of the enzyme for subsequently bound molecules of substrate, while increasing the catalytic rate: i.e. substrate binding displays negative cooperativity while catalytic activity displays positive cooperativity. This explanation of the experimental data has been proposed for the F-type ATPases, where substrate-binding as well as catalytic studies have been performed (Gresser *et al.*, 1982; Grubmeyer *et al.*, 1982; Cross *et al.*, 1982; Roveri & Calcaterra, 1985).

The models that have been developed to explain the occurrence of different K_m and V_{max} values for ATP hydrolysis in F-type ATPases are controversial. These effects have been explained by postulating that the enzyme has three catalytic sites (Gresser *et al.*, 1982; Roveri & Calcaterra, 1985), however, this model has been criticised by Berden *et al.* (1991), who have proposed an alternative model based on the existence of only two catalytic sites.

Source (type)	K_m (μM)			V_{max} (Units/mg)			Reference
	1.7	250		2.2	55		(a)
Beef heart mitochondria (F)	10^{-6}	30	150	10^{-4}	300	600 (*)	(b), (c)
	4	40	500	2	6	42	(d)
Clathrin-coated vesicles (V)	83	790		2.1	5.8		(e)
Chromaffin granules (V)	1.4	174		0.032	1.3		(f)
	5	30	300	0.47	1.2	2.6	(g)
<i>Kalanchoë</i> tonoplast (V)	1.9	776		0.27	3.65		(h)

Table 4.6. Comparison between kinetic parameters obtained for ATP hydrolysis activity of ATPases from different sources. References: (a) Gresser *et al.* (1982); (b) Grubmeyer *et al.* (1982); (c) Cross *et al.* (1982); (d) Roveri & Calcaterra (1985); (e) Arai *et al.* (1989); (f) Pérez-Castiñeira (1991); (g) Hanada *et al.* (1990); (h) Warren *et al.* (1992).

(*) Data in s^{-1}

Most of the kinetic work done on the *Kalanchoë* ATPase concentrated on H⁺-translocation. The binding of substrate by the enzyme was not studied independently and it was therefore impossible to draw any conclusions about the structure of the ATPase from the kinetic data alone. Although a structural model capable explaining the observed kinetic effects might be developed, such models are highly theoretical unless supported by direct evidence relating to the number and type of nucleotide-binding sites. The only independent measurement of MgATP binding so far reported is that of Ward *et al.* (1992), who deduced a K_d of 34 μ M from the effect of MgATP on dissociating the extramembrane subunits of the H⁺-ATPase from oat-root tonoplast.

The investigation of the regulatory properties of the ATPase produced the most surprising results. In the absence of inhibitors, the enzyme displayed a near Michaelis-Menten dependence on [MgATP²⁻]. However, in the presence of nucleoside diphosphates, the binding of ATP became cooperative. Calculation of Hill parameters for this data showed that $n_h < 1$ in the absence of inhibitors, but increased in the presence of ADP, GDP or IDP (see Table 4.3). The value of n_h gives an idea of the deviation from Michaelis-Menten kinetics, values below 1 suggesting negative cooperativity and values above 1 suggesting positive cooperativity. Therefore, the *Kalanchoë* ATPase displays positive cooperativity in the presence of purine nucleoside diphosphates.

ADP proved to be the most effective of the nucleoside diphosphate inhibitors, and inhibition of the H⁺-translocation activity of the reconstituted ATPase was studied over a range of ADP concentrations. However, calculation of the Hill parameters for this large data set gave $n_h > 1$ in the

absence of ADP, and this value did not greatly increase with increases in ADP concentration (see Table 4.4). This contradicts the findings from the smaller data set with different nucleoside diphosphates (Table 4.3). For comparison Table 4.4 also contains data obtained from ADP inhibition studies on the reconstituted ATPase from bovine chromaffin granules (Pérez-Castiñeira, 1991). For the chromaffin granule enzyme the value of n_h was found to increase with increasing ADP concentration, as would be expected with a K-type allosteric inhibitor. The finding that, for the *Kalanchoë* enzyme, the value of n_h appears to be unaffected by increases in ADP concentration, may be due to the fact that the activity of the tonoplast ATPase was for technical reasons assayed over a smaller substrate concentration range than the chromaffin granule enzyme (10 μ M - 1 mM for the plant ATPase as opposed to 1 μ M - 1 mM for the animal ATPase). Since the *Kalanchoë* ATPase was not assayed at substrate concentrations $< 10 \mu$ M for the ADP inhibition study, then the absence of these points from the data set may affect the final values obtained for n_h when the Hill parameters are calculated. When the data for individual ADP concentrations are considered, it can be seen that there are not many points when $[ADP] = 0$ (Fig. 4.8a), and that when $[ADP] = 500 \mu$ M (Fig. 4.8f) the enzyme does not reach saturation, therefore the trend in n_h may be obscured. Overall the model predicts increases in n_h with increasing inhibitor concentrations. In a separate data set for ADP inhibition of the *Kalanchoë* enzyme, including $[MgATP]$ down to 1.5 μ M, the enzyme showed cooperativity at $[MgATP] < 10 \mu$ M, in the presence of ADP. However, calculation of the Hill parameters for this second ADP inhibition data set also gave $n_h > 1$ in the absence of ADP, and again this value did not greatly increase with increases in ADP

concentration (data not shown). Thus fitting the ADP inhibition data to the Hill equation appears to obscure the trend of increasing positive cooperativity that the *Kalanchoë* ATPase displays in the presence of increasing concentrations of ADP.

In order to explain the effects produced by the inhibitors, particularly ADP, a theoretical model was developed. The concerted allosteric model of Monod *et al.* (1965) was chosen for this purpose because it is relatively simple and makes structural predictions about the enzyme. An attempt was then made to fit the experimental data to the mathematical expressions derived from the model.

If it was assumed that the number of binding sites for substrate and inhibitor were the same, a poor fit of the model to the experimental data was consistently obtained. This model also consistently underestimated V_{\max} and gave a higher value of the conformational equilibrium constant (L) than would have been expected. To allow for the fact that the number of inhibitor binding sites might be different from the number of catalytic sites, a new parameter (m) distinct from n was introduced. When m was fixed at 1 and n allowed to 'float', a very good overall fit was obtained; however, the calculated value of n was 1.58, not close to an integral value; moreover, the extremely large value of L obtained was not consistent with the lack of cooperativity in the absence of ADP.

The best overall fit was obtained with the assumption that there were 2 substrate binding sites and a single inhibitor binding site. This fitting also produced more reasonable parameter values which were in accordance with the assumptions of the model, except that L was still large. This result was only slightly better than that obtained by postulating three binding sites for the substrate and one for the inhibitor: this produced a value of L close to 1. However, in both cases the standard error in the value of L was almost as

large as the value of the parameter itself, so this is not criterion for judging the success of a particular model.

The concerted allosteric model appears to be able to describe the properties of the *Kalanchoë* ATPase fairly well. The model predicts the existence of a single high-affinity inhibitor-binding site and several (2 or 3) substrate-binding sites. The enzyme can exist in one of two states, either catalytically active (R) or inactive (T). In the R state the inhibitor site is not available and the substrate binding sites have high affinity for the substrate, while in the T state the high-affinity inhibitor binding site is available and there is a decrease in the affinity of the substrate binding sites for the substrate. In the absence of any inhibitors the R state is favoured relative to the T state (in other words the conformation equilibrium constant L is small), and this accounts for the near Michaelis-Menten kinetics observed under these circumstances.

In considering the conclusions drawn from the model, the important assumptions that are made in deriving it have to be taken into account. Firstly, the “concerted” allosteric model explains cooperative interactions in terms of identical subunits that exist in two (or more) states in equilibrium, the substrates or effectors exerting their effects by displacing the equilibrium toward the state to which they bind with higher affinity. It is apparent that this model does not account for cooperative interactions (either positive or negative) within each of the possible states, so the existence of binding sites with different affinities for substrates or effectors within a given state cannot be explained either. However, the ATP hydrolysis data do suggest negative cooperativity between the catalytic sites of the enzyme.

The theoretical model might be improved by considering some catalytic activity for the T-state and more possible sub-states for the enzyme within the R-state, thus accounting for interactions between subunits in the active form

of the enzyme. Both considerations would have meant the inclusion of new parameters and further complication of the mathematical equations. This was not considered justified on the basis of the experimental data, as the large number of disposable constants makes data fitting difficult even with the restricted models.

There is a further important reservation: all kinetic models are postulated in terms of substrate and inhibitor-binding, that is, the Hill equation and all the equations relating to the MWC allosteric model give values of saturation functions (Y_s) for proteins (with or without catalytic activity). The catalytic rate has been assumed to be proportional to this function, i.e., $v_o = k_{cat}Y_s$. The experiments performed give information on initial velocities of reaction and the value of v_o/V_{max} is strictly proportional to the saturation function only if a Michaelis equilibrium applies, that is, if the substrate-binding equilibrium is not affected by the rate of formation of products. Therefore the data gave only partial, although useful information about the binding properties of the *Kalanchoë* H^+ -ATPase.

In order to draw definite conclusions about the functional properties of vacuolar ATPases as well as their physiological and structural implications, proper ligand-binding studies must be carried out, as has already been done for F-type ATPases (Grubmeyer *et al.*, 1982; Cross *et al.*, 1982). In particular, estimation of the binding constants for nucleoside diphosphates might be attempted by the use of equilibrium dialysis or a similar technique. Independent estimation of binding constants for MgATP is difficult, since MgATP is a substrate. Similarly, determination of the value of L requires a means of measuring the conformational equilibrium directly, which is probably not feasible with a membrane-bound enzyme.

Interestingly, kinetic and regulatory studies performed on the chromaffin

granule ATPase (Pérez-Castiñeira, 1991) produced similar results to those obtained for the *Kalanchoë* enzyme. The data from the experiments performed on the chromaffin granule enzyme, like that from the work on the *Kalanchoë* ATPase, could be best fitted to a theoretical model that assumed a single inhibitor-binding site and two substrate binding sites. This suggests the model that has been developed could be of general applicability to all V-type ATPases.

CHAPTER 5

5. Immunological investigation of V-type ATPases

5.1. Introduction

Information from both immunological studies (Manolson *et al.*, 1989; Konishi *et al.*, 1990) and gene sequencing (Gogarten *et al.*, 1989) suggests that there is a common evolutionary ancestor for all V-type ATPases. These studies have also shown that the V-type ATPases have a discernible structural relationship to the F-type ATPases of eubacteria, mitochondria and chloroplasts; however, they are rather more closely related to the plasma membrane H⁺-ATPases of archaeobacteria (see Chapter 1, section 1.3 for detailed discussion).

The existence of multiple isoforms of the subunits of F-type (Walker *et al.*, 1989) and P-type (Lingrel *et al.*, 1990) ATPases in mammals is well known. Because of their widespread distribution and diverse intracellular locations, it might be predicted that multiple isoforms of at least some of the subunits of V-type ATPases will be found, and evidence for this is already accumulating. To-date two isoforms of the non-catalytic nucleotide binding 57 kDa subunit of the human V-type ATPase have been identified (Südhof *et al.*, 1989; Bernasconi *et al.*, 1990). Heterogenous forms of the 56 kDa and 31 kDa subunits from the bovine kidney vacuolar ATPase have also been found (Wang & Gluck, 1990). There is also immunological evidence that the membrane distribution of the vacuolar ATPase within the kidney is dependent upon which isoform of the 31 kDa subunit is being expressed (Hemken *et al.*, 1992).

To further the investigation of structural similarities between the *Kalanchoë* tonoplast ATPase and animal endomembrane ATPases, rabbit

polyclonal antibodies were raised to the 72 kDa and 57 kDa subunits of the plant enzyme. It was also intended to use these antibodies to search for isoforms of the V-type ATPase from a particular animal, and to this end the Triton X-114 fractionation procedure was used to produce V-ATPase-enriched fractions from microsomal membranes prepared from several bovine tissues.

5.2. Materials and Methods

5.2.1. Sources of the vacuolar ATPases

Tonoplast membranes from *Kalanchoë daigremontiana* were prepared as described in Chapter 2, section 2.3. Microsomal membranes from *Dictyostelium discoideum* were a gift from Dr E.K. Rooney and Dr J.D. Gross, University of Oxford, Oxford, U.K. Microsomal membranes from *Saccharomyces pombe* were a gift from Dr J.G. Pryde, ICRF, Lincoln's Inn Fields, London, U.K. Human chromaffin granule membranes were prepared from a pheochromocytoma using a published method (Apps *et al.*, 1980). The H⁺-ATPase was purified from human chromaffin granule membranes by fractionation with Triton X-114 as described by Pryde & Phillips (1986) (see below as well). The purification of vacuolar ATPases from several bovine sources is described below.

5.2.2. Purification of bovine vacuolar ATPases

5.2.2.1. Purification of the chromaffin-granule H⁺-ATPase

Chromaffin granule membranes were purified from fresh bovine adrenal medullae according to a published procedure (Apps *et al.*, 1980) and stored at -20°C in 10 mM Hepes/NaOH (pH 7.4), 0.1 mM EDTA, 1 mM DTT.

Chromaffin-granule membranes were fractionated with Triton X-114 according to published methods (Pryde & Phillips, 1986; Pérez-Castiñeira & Apps, 1990). After thawing, the membranes were sedimented (100,000 rev/min, Beckman TL-100.3 rotor, $g_{av} = 340,000$, 10 min). The membrane pellet was resuspended in 0.15 M KCl, 10 mM Tris/HCl (pH 7.4), 1 mM EDTA, 1 mM DTT, with gentle homogenization in a glass homogeniser. Triton X-114 was added so that the final concentrations were: detergent, 20 mg/ml; membranes, 4 mg of protein per ml. After 5 min at 0°C, the white precipitate was removed by centrifugation as described above and washed once with 2 % (w/v) Triton X-114 in the same buffer. This Triton-insoluble material was highly enriched in V-type ATPase and was used for immune blotting.

5.2.2.2. Purification of the kidney H⁺-ATPase

Kidney microsomes were prepared by an adaptation of a published procedure (Gluck & Al-Awqati, 1984). All solutions contained 10 mM Tris-HCl (pH 8.0), 1 mM NaHCO₃, 1 mM EDTA, 1 mM DTT, plus sucrose at the required concentration. Bovine renal medullary tissue was minced and homogenised in 0.25 M sucrose, and centrifuged for 10 min at $g_{av} = 1600$.

After centrifugation of the supernatant for 60 min at $g_{av} = 41,000$, crude microsomes were removed from the surface of the pellet by swirling, collected by centrifugation, resuspended in 0.25 M sucrose, overlaid onto "step gradients" of 0.7, 1.0 and 1.5 M sucrose, and centrifuged for 5 h at 35,000 rev/min in a swingout rotor (Beckman SW41). The microsomal fraction was collected from the 0.7 M/1.0 M sucrose interface, diluted, centrifuged and finally resuspended in 0.25 M buffered sucrose containing the protease inhibitors benzamidine (2 mM), pepstatin (5 μ g/ml), leupeptin (5 μ g/ml).

Fractionation of the kidney microsomes with Triton X-114 was essentially as described for chromaffin granule membranes (see section 5.2.2.1), except that the final concentration of detergent was 17.5 mg/ml. The Triton-insoluble material was used for immune blotting.

5.2.2.3. Purification of the retinal H^+ -ATPase

Bovine retinal microsomes were prepared by the following method. Retinal membranes were removed from eyes and washed with 10 mM Tris/HCl (pH 7.4), 150 mM KCl. The washed membranes were homogenised in 10 mM Hepes/NaOH (pH 7.2), 0.32 M sucrose and centrifuged (4,000 rev/min, Beckman JA 20 rotor, $g_{av} = 1250$) for 5 min. The supernatant was collected and kept on one side. The pellet was resuspended in homogenisation buffer, the volume of buffer added being equal to that of the pellet. The retinal membranes were re-homogenised and then centrifuged (11,000 rev/min, Beckman JA 20 rotor, $g_{av} = 14,600$) for 5 min. The pellet was discarded and the supernatant was pooled with that from the first homogenisation. The supernatant was centrifuged (35,000

rev/min, Beckman Ti 45 rotor, $g_{av} = 100,000$) for 1 h to pellet the microsomal membranes. The membranes were resuspended in a minimal volume of buffer containing 10 mM Hepes/NaOH (pH 7.2), 0.32 M sucrose, 1 mM EDTA, 1 mM DTT. The microsomes were further purified by centrifugation on 5 ml gradients of 0.4-2.0 M sucrose. Aliquots of 0.6 ml of the microsomal membranes were layered onto these gradients, which were centrifuged for 5 h at 45,000 rev/min (Beckman SW50.1; $g_{av} = 190,000$). After centrifugation, the gradients were fractionated into 0.5 ml samples. The fractions were then diluted sixfold and then centrifuged (100,000 rev/min, Beckman TL 100.3 rotor, $g_{av} = 340,000$, 10 min) to pellet the membranes. The membranes were resuspended in 200 μ l of buffer containing 10 mM Hepes/NaOH (pH 7.2), 1 mM EDTA, 1 mM DTT. To identify the fractions containing the retinal H^+ -ATPase, samples from each fraction were western-blotted and then probed with antibodies directed against the 57 kDa subunit of the *Kalanchoë* ATPase. Those fractions which were found to contain the vacuolar ATPase were kept and pooled.

Fractionation of the retinal microsomes with Triton X-114 was essentially as described for chromaffin granule membranes (see section 5.2.2.1) using 20 mg/ml Triton X-114. The Triton-insoluble material was used for immune blotting.

5.2.2.4. Purification of the pituitary H^+ -ATPase

Pituitary microsomes were prepared by the following method. Two bovine pituitaries were minced in 10 mM Hepes/NaOH (pH 7.2), 1 mM EDTA containing 2 mM benzamidine, 20 μ M TLCK, 20 μ M TPCK, 50 μ M PMSF,

pepstatin (2 µg/ ml) and leupeptin (2 µg/ml). The homogenate was centrifuged (4,000 rev/min, Beckman JA 20 rotor, $g_{av} = 1250$) for 10 min. The pellet was discarded and the supernatant centrifuged (15,000 rev/min, Beckman JA 20 rotor, $g_{av} = 17,000$) for a further 20 min. The second pellet was retained and the supernatant centrifuged (50,000 rev/min, Beckman SW50.1 rotor, $g_{av} = 230,000$) for 1h. The pellets from the second and third centrifugation steps were resuspended in buffer and centrifuged (50,000 rev/min, Beckman SW50.1 rotor, $g_{av} = 230,000$) for 1h. This gave a large granule fraction and the microsomes.

Fractionation of the pituitary microsomes with Triton X-114 was essentially as described for chromaffin granule membranes (see section 5.2.2.1), except that the final concentration of detergent was 17.5 mg/ml. The Triton-insoluble material was used for immune blotting.

5.2.3. Two-dimensional polyacrylamide gel electrophoresis

Two-dimensional electrophoresis was performed using the Hoefer "Tall Mighty Small" mini-gel system. The protocol is based on the method of O'Farrell (1975). The following solutions were used:

45 % (w/v) acrylamide, 0.6 % (w/v) bis-acrylamide (deionised with mixed bed resin)

10 % (w/v) Nonidet P-40 (deionised)

40 % (w/v) Ampholines, pH ranges 3-10 and 9-11

0.2 % (v/v) phosphoric acid (anode buffer)

0.6 % (v/v) ethanolamine (cathode buffer)

Sample buffer (freshly made up) :

0.57 g urea

0.20 ml Nonidet P-40

0.30 ml water

0.05 ml ampholine

0.05 ml 0.5 M DTT

2 μ l bromophenol blue (0.5 mg/ml in water/ methanol 1:1)

Soaking buffer:

30 ml 10 % (w/v) SDS

10 ml 0.5 M Tris-HCl (pH 6.5)

10 ml glycerol

1 ml 0.5 M DTT

0.1 ml bromophenol blue (0.5 mg/ml)

Water to 100 ml

Agarose:- using high grade, low melting point agarose, make up a 1% (w/v) solution in soaking buffer (about 2 ml required for each gel). Keep at 100°C.

5.2.3.1. First-dimension: electrofocusing

Isoelectric focusing gels were made in glass tubes designed for use with the Hoefer "Tall Mighty Small" mini-gel apparatus. The required number of tubes were set up in the casting cup, lashed to the support post with a rubber band and then 5.5 ml of water was put in the outer chamber. The following mixture of 2.38 g urea, 0.42 ml acrylamide solution, 0.80 ml Nonidet P-40 and 1.16 ml water, was made up in a Quick-fit tube. The tube was shaken gently until the urea had completely dissolved, then the solution was briefly

degassed using a water pump. To the degassed solution, 200 μ l ampholine (pH range 3-10) and 50 μ l ampholine (pH range 9-11) were added.

Polymerization was started with the addition of 20 μ l fresh 10 % (w/v) ammonium persulphate. The mix was immediately added to the casting cup, which was then lowered into the outer chamber. Using a 25 ml syringe with a very thick needle, 17.5 ml water was gently added to the outer chamber, so that the acrylamide mix was displaced upwards into the tubes. When set, the gels were carefully removed from the cup by cutting away the mass of polymerized acrylamide at the bottom, ensuring that the acrylamide in the tubes was intact.

To set the gels up ready for running, the tubes were pushed into the tube gel adaptor, and any unused gel slots were stoppered. The adaptor was greased and clipped to the upper buffer chamber and then snapped into the lower buffer chamber. The upper chamber and the tops of the gel-tubes were filled with cathode buffer. The lower chamber was filled with anode buffer. The tubes were checked to ensure there were no bubbles lurking in the tops of the tubes, or under the lower ends of the gels.

The samples were spun down in a Beckman TL 100 bench-top ultra-centrifuge and then dissolved directly in sample buffer. The samples containing up to 50 μ g protein (max. volume 25 μ l) were carefully loaded onto the tops of the gels. To focus, the gels were run for 2-4 h at 500 V. At the end of the run the bromophenol blue appeared as a sharp yellow band at the bottom of the gel. After running, the gels were removed from their tubes and immersed in soaking buffer for about 10 min before running the second dimension. Alternatively the gels could be stored deep-frozen at this stage for use later.

5.2.3.2. Second dimension: SDS-polyacrylamide electrophoresis

The mini-gel cassettes were made up (as described in Chapter 2, section 2.9.1) using thick (1.5 mm) spacers. The gels were poured right up to the lip of the cassette - no stacking gel was required. Once set, the unpolymerized acrylamide was washed from the tops of the gels. The slab gels were set up as usual. Each first-dimension tube gel was placed onto the top of a slab gel. After checking that there were no bubbles under the gels, they were sealed on with hot low-melting point agarose (1 %) boiled in soaking-buffer. Once the agarose had set, the gels were run in the normal way, as described in Chapter 2, section 2.2.7.

5.2.4. **IgG preparation by octanoic acid precipitation**

To one volume of serum, two volumes of 60 mM acetate buffer, pH 4.0 were added and mixed using a magnetic stirrer. Octanoic acid was added dropwise to the serum/ acetate buffer solution while stirring quite vigorously, with 75 μ l of octanoic acid being added for every 1 ml of serum used. After all the octanoic acid had been added, the mixture was stirred vigorously for a further 30 minutes.

The solution was centrifuged for 20 min at 15,000 rev/min in a Beckman JA 20 rotor. The supernatant was then decanted off and dialysed against 20 mM phosphate buffer pH 6.2 at 4°C for 48 hours. Any precipitate which formed was removed by centrifugation. The supernatant was concentrated in an Amicon filter to give a small volume of high protein concentration. The purified IgG fraction was then aliquoted and stored at -20°C until required.

The measurement of proton-translocation and immuno-blotting techniques are described in Chapter 2, sections 2.2.5. and 2.2.8., respectively.

5.3. Characterisation of the antibodies raised to the 57 kDa and 72 kDa subunits of the *Kalanchoë* vacuolar ATPase

The antibodies were raised according to the protocol described in Chapter 2 (see section 2.9). Two rabbits were immunised for each of the subunits, producing four antisera, two directed against the 57 kDa subunit (antisera numbers 321 and 322) and two directed against the 72 kDa subunit (antisera numbers 320 and 323). To characterise these antibodies, nitrocellulose strips, onto which tonoplast protein had been transferred after electrophoretic separation, were incubated with dilutions of the antisera ranging from 1:200 to 1:3200. These "strip-blot" were then visualised using the biotin -¹²⁵I streptavidin system (see Fig. 5.1). From Fig. 5.1 it appears that all four antisera reacted strongly with tonoplast protein even at high dilution. Both of the 72 kDa antisera were very specific, binding strongly to a single band at high dilution. Of the 57 kDa antisera 322 bound most strongly, but showed a high degree of non-specific binding even at high dilution; whereas, 321 while not binding as strongly shows greater specificity, binding to a single band at high dilution.

To confirm that the observed antibody binding was genuine, nitrocellulose strips blotted with tonoplast protein were incubated with pre-immune serum from each of the rabbits that had been immunised with the 57 kDa and 72 kDa subunits. These control strips were then compared with strips incubated in each of the four antisera (Fig 5.2). From Fig 5.2 it appears that only 322 pre-immune serum showed any significant non-specific binding in

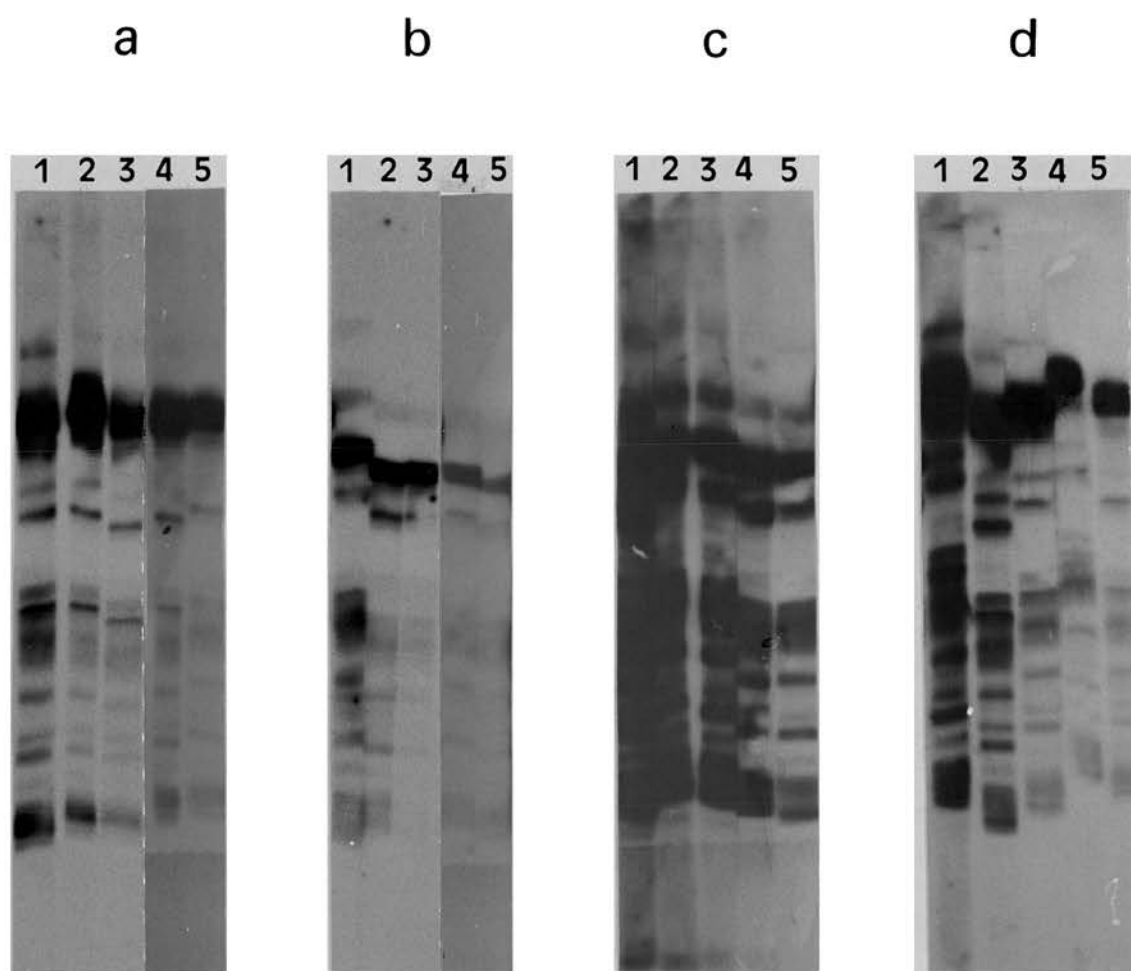


Fig. 5.1. Characterisation of 57 kDa and 72 kDa antisera. Tonoplast protein (500 μ g) was electrophoresed and blotted onto nitrocellulose. The blot was cut into strips (approx. 30 μ g protein per strip) which were then probed with following antisera: 320 anti-72 kDa serum (a), 321 anti-57 kDa serum (b), 322 anti-57 kDa serum (c) and 323 anti-72 kDa serum (d) at dilutions of: 1:200 (lane 1), 1:400 (lane 2), 1:800 (lane 3), 1:1600 (lane 4), 1:3200 (lane 5), followed by biotinylated anti-rabbit goat IgG and 125 I-streptavidin.

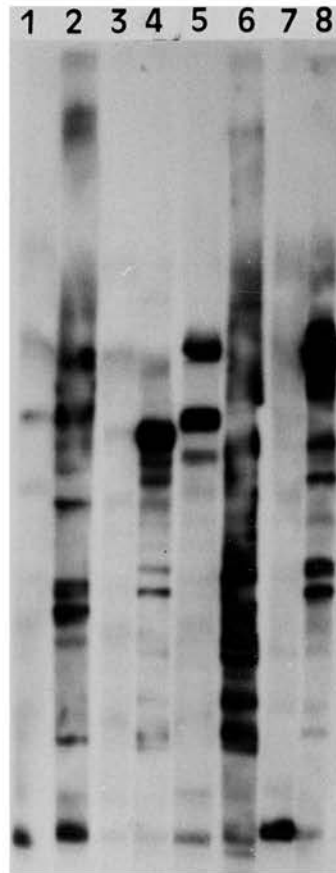


Fig. 5.2. Specificity of the anti-57 kDa and anti-72 kDa sera for the tonoplast ATPase. Tonoplast protein (500 μ g) was electrophoresed and blotted onto nitrocellulose. The blot was cut into strips (approx. 30 μ g protein per strip) which were then probed with either pre-immune sera diluted 1:100, or the corresponding antisera diluted 1:1600, followed by biotinylated anti-rabbit goat IgG and 125 I-streptavidin. The antisera tested were: 320 pre-immune serum (lane 1), 320 anti-72 kDa serum (lane 2), 321 pre-immune serum (lane 3), 321 anti-57 kDa serum (lane 4), 322 pre-immune serum (lane 5), 322 anti-57 kDa serum (lane 6), 323 pre-immune serum (lane 7) and 323 anti-72 kDa serum (lane 8).

the region where the 57 kDa and 72 kDa antigens are bound to the nitrocellulose.

5.4. Cross-reactivity of antibodies raised to the 57 kDa and 72 kDa subunits of the *Kalanchoë* vacuolar ATPase with the corresponding polypeptides of V-type ATPases from different sources

The cross-reactivity of antibodies raised to V-type ATPase subunits from one source with the corresponding subunits of V-type enzymes from widely different sources is well documented (Bowman *et al.*, 1986; Mandala & Taiz, 1986; Moriyama & Nelson, 1989^{b,c}; Manolson *et al.* 1989). Therefore it was unsurprising to find that the antibodies raised to the 57 kDa and 72 kDa subunits of the *Kalanchoë* enzyme cross-reacted to the corresponding subunits of V-type ATPases from a range of sources (Fig. 5.3). It can be seen that the antibodies raised against the *Kalanchoë* ATPase subunits showed significant cross-reactivity with the corresponding subunits from mammalian V-type ATPases. The 57 kDa antibodies reacted particularly well with both the bovine and human chromaffin granule enzymes (Fig. 5.3a). The 72 kDa antibodies appeared to be less specific for the corresponding subunit in mammalian enzymes, binding to polypeptides of molecular mass less than 72 kDa in addition to the 72 kDa subunit, whereas the 57 kDa antibodies bound exclusively to the 57 kDa polypeptide in the mammalian preparations. No cross-reactivity was observed with membranes from *Saccharomyces pombe* or the amoeba *Dictyostelium discoideum*.

When this same group of vacuolar enzymes were immunoblotted with antibodies raised against the 120 kDa subunit of the bovine chromaffin granule ATPase, the 120 kDa subunits of all the mammalian enzymes

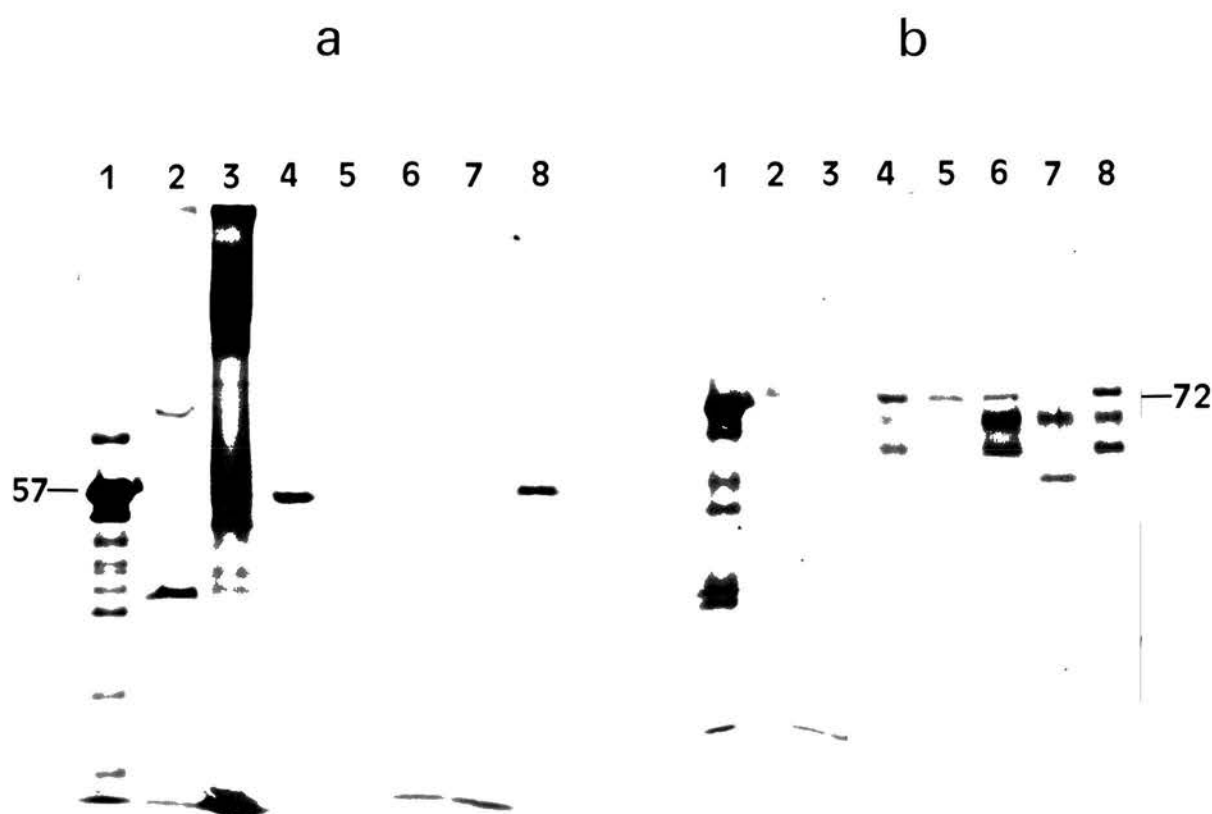


Fig. 5.3. Immunological cross-reactivity of antibodies directed against the 57 kDa and 72 kDa subunits of the *Kalanchoë* tonoplast H^+ -ATPase with plant, animal and fungal endomembrane H^+ -ATPases. Lane 1, *Kalanchoë* tonoplast membranes; lane 2, *Dictyostelium* microsomes; lane 3, *Saccharomyces pombe* microsomes; lane 4, Triton X-114 purified bovine chromaffin granule H^+ -ATPase; lane 5, Triton X-114 purified bovine kidney microsome H^+ -ATPase; lane 6, Triton X-114 purified bovine retinal microsome H^+ -ATPase; lane 7, Insulinoma granule membranes; lane 8, Triton X-114 purified human chromaffin granule H^+ -ATPase. 20 μ g protein was loaded onto each lane (except lane 1, 5 μ g). The samples were subjected to SDS-PAGE using a mini-gel apparatus, transferred to nitrocellulose and probed with: (a) a 1:500 dilution of 322 anti-*Kalanchoë* 57 kDa serum; (b) a 1:200 dilution of 323 anti-*Kalanchoë* 72 kDa serum, followed by horse-radish peroxidase anti-rabbit goat IgG and visualisation using the ECL system.

reacted strongly with these antibodies, but more interestingly, a polypeptide of similar molecular weight present in tonoplast membranes also appeared to cross-react with the 120 kDa antibodies (see Fig. 5.4). This is in contrast to a previous result in which fractions from each stage of the purification and reconstitution procedure for the *Kalanchoë* ATPase were immunoblotted against the same 120 kDa antiserum and no evidence was found for the existence of a subunit with a molecular mass in this range (see Chapter 3, Fig. 3.12b). Like the anti-57 kDa and anti-72 kDa sera, the anti-120 kDa serum did not recognise any antigen in the *Dictyostelium* or *S. pombe* membranes.

Closer examination of Fig. 5.3a reveals slight differences in electrophoretic mobility between the 57 kDa subunits of the bovine V-type ATPases from chromaffin granules, kidney microsomes and retinal microsomes, suggesting the possibility of isoforms of 57 kDa subunit in the bovine vacuolar ATPase. This can be seen more clearly in Fig. 5.5a in which the anti-57 kDa serum recognises a doublet in the kidney fraction. This observation has been reported elsewhere (Gillespie *et al.*, 1991: see Appendix 2 and compare Fig. 1 of the paper with Fig. 5.4a).

5.5. Immunological investigation of possible heterogeneity of the 57 kDa and 72 kDa subunits of the bovine vacuolar ATPase

To investigate the possible existence of isoforms of the bovine vacuolar ATPase, partially purified ATPase preparations from chromaffin granules, kidney microsomes, pituitary microsomes and retinal microsomes were analysed by two-dimensional immunoblotting.

The ATPase from each source was purified by fractionation of the

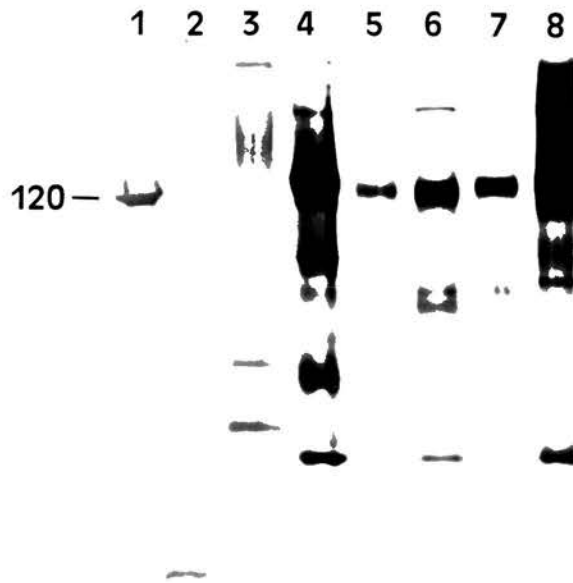


Fig. 5.4. Immunological cross-reactivity of antibodies directed against the 120 kDa subunit of the bovine chromaffin granule H^+ -ATPase with plant, animal and fungal endomembrane H^+ -ATPases. Lane 1, *Kalanchoë* tonoplast membranes; lane 2, *Dictyostelium* microsomes; lane 3, *Saccharomyces pombe* microsomes; lane 4, Triton X-114 purified bovine chromaffin granule H^+ -ATPase; lane 5, Triton X-114 purified bovine kidney microsome H^+ -ATPase; lane 6, Triton X-114 purified bovine retinal microsome H^+ -ATPase; lane 7, Insulinoma granule membranes; lane 8, Triton X-114 purified human chromaffin granule H^+ -ATPase. 20 μ g protein was loaded onto each lane. The samples were subjected to SDS-PAGE using a mini-gel apparatus, transferred to nitrocellulose and probed with a 1:200 dilution of chromaffin granule anti-120 kDa serum, followed by horse-radish peroxidase anti-rabbit goat IgG and visualisation using the ECL system.

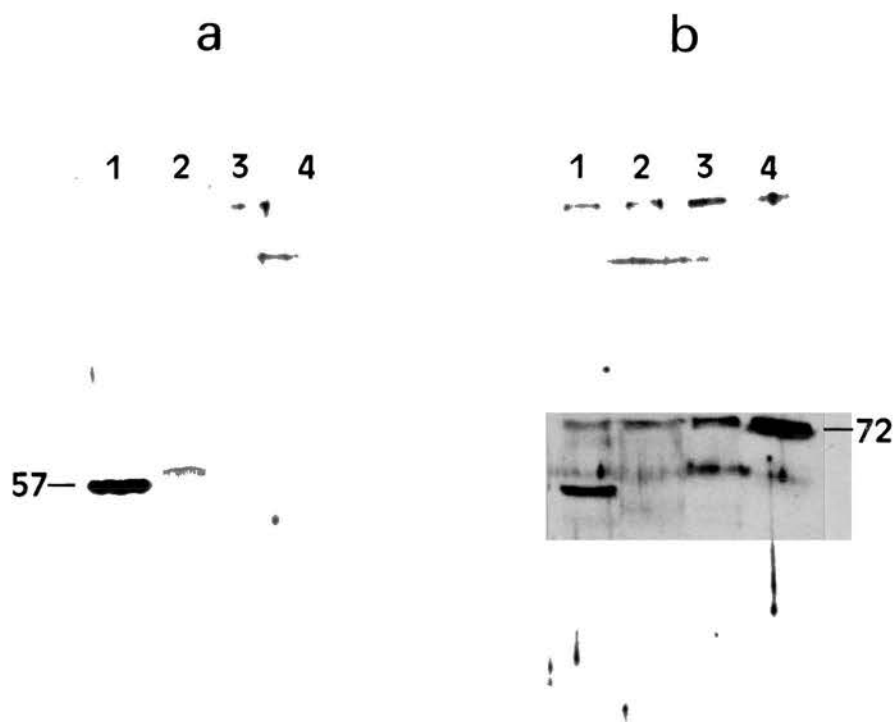


Fig. 5.5. Immunological cross-reactivity of antibodies directed against the 57 kDa and 72 kDa subunits of the *Kalanchoë* tonoplast H^+ -ATPase with H^+ -ATPases from four different bovine sources. (a) lane 1, Triton X-114 purified chromaffin granule H^+ -ATPase; lane 2, Triton X-114 purified kidney microsome H^+ -ATPase; lane 3, Triton X-114 purified pituitary microsome H^+ -ATPase; lane 4, Triton X-114 purified retinal microsome H^+ -ATPase. (b) lane 1, Triton X-114 purified retinal microsome H^+ -ATPase; lane 2, Triton X-114 purified pituitary microsome H^+ -ATPase; lane 3, Triton X-114 purified kidney microsome H^+ -ATPase; lane 4, Triton X-114 purified chromaffin granule H^+ -ATPase. 20 μ g protein was loaded onto each lane, the samples were then subjected to SDS-PAGE using a mini-gel apparatus, transferred to nitrocellulose and probed with: (a) a 1:500 dilution of 322 anti-*Kalanchoë* 57 kDa serum; (b) a 1:400 dilution of 323 anti-*Kalanchoë* 72 kDa serum, followed by horse-radish peroxidase anti-rabbit goat IgG and visualisation using the ECL system.

membranes with the detergent Triton X-114. A monoclonal antibody against bovine dopamine- β -monooxygenase (DBH; EC 1.14.17.1) was available, and small quantity of this protein was added to each of the purified enzyme preparations (1 μ g DBH per 5 μ g ATPase protein) to act as a marker. The samples were then subjected to two-dimensional gel electrophoresis followed by electro-blotting onto nitrocellulose. These two-dimensional blots were then probed with the following antibodies; anti-*Kalanchoë* 57 kDa polyclonal; anti-DBH monoclonal and anti-*Kalanchoë* 72 kDa polyclonal, in succession. After incubation with each of these antibodies the blots were developed using the ECL system (see Chapter 2, section 2.8.2). After developing, the blots were washed with 0.05 % (w/v) sodium azide to inactivate the horse-radish peroxidase activity of the conjugated second antibody, before they were incubated with the next primary antibody. The blots obtained for each ATPase are shown in Figs. 5.6 and 5.7.

To produce the pictures shown in Figs. 5.6 and 5.7 the autoradiographs of the anti-57 kDa and anti-72 kDa blots respectively were superimposed over that of their corresponding DBH blots. By comparing the position of the 57 kDa and 72 kDa spots of the four bovine ATPases relative to the DBH spot, it is possible to determine whether there are any isoforms of these subunits. In Fig. 5.6b it can be seen that there is heterogeneity of the 57 kDa spot of the kidney ATPase, while the adrenal, pituitary and retinal ATPases show predominantly a single 57 kDa polypeptide. The predominant 57 kDa spot of the kidney ATPase has a larger pI than the 57 kDa spots of the other three bovine ATPases. This suggests that distinct isoforms of the 57 kDa subunit are present in the kidney ATPase, whereas a single isoform of the 57 kDa subunit, different from that found in kidney, is present in the adrenal, pituitary and retinal ATPases. Alternatively, the apparent difference in the pI of the

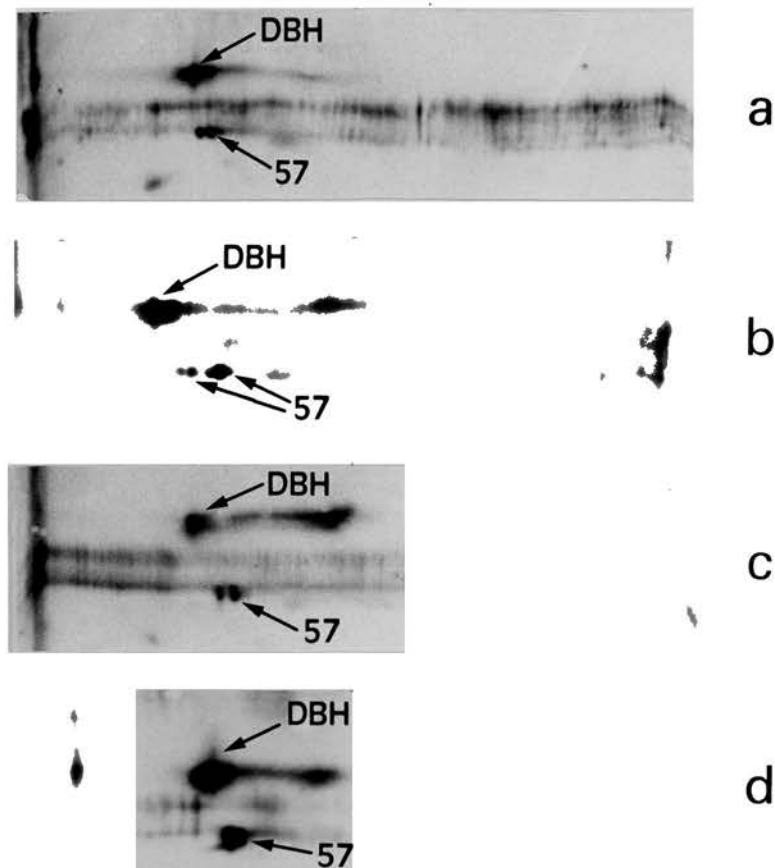


Fig. 5.6. Two-dimensional immunoblots of H^+ -ATPases purified from four bovine sources: (a) chromaffin granules, (b) kidney microsomes, (c) pituitary microsomes, (d) retinal microsomes. The ATPases were purified by fractionating the membranes with Triton X-114. 1 μ g DBH was added to every 5 μ g ATPase protein, to act as a marker. The samples were then subjected to two-dimensional gel electrophoresis followed by blotting onto nitrocellulose. Each blot was then probed with the following antisera: firstly, a 1:400 dilution of 322 anti-*Kalanchoë* 57 kDa serum, followed by a 1:20 dilution of anti-DBH monoclonal supernatant. Incubation of the blots with primary antibody was followed by either horse-radish peroxidase conjugated anti-rabbit goat IgG (for *Kalanchoë* Ab) or by horse-radish peroxidase conjugated anti-mouse rabbit IgG (for DBH monoclonal) and then visualisation by the ECL system. Figs. 5.6a-d were produced by super-imposing the autoradiograph of the anti-57 kDa blot over that of the DBH blot. In each case only the relevant part of the blot is shown, but there was no significant immunoreactivity elsewhere.

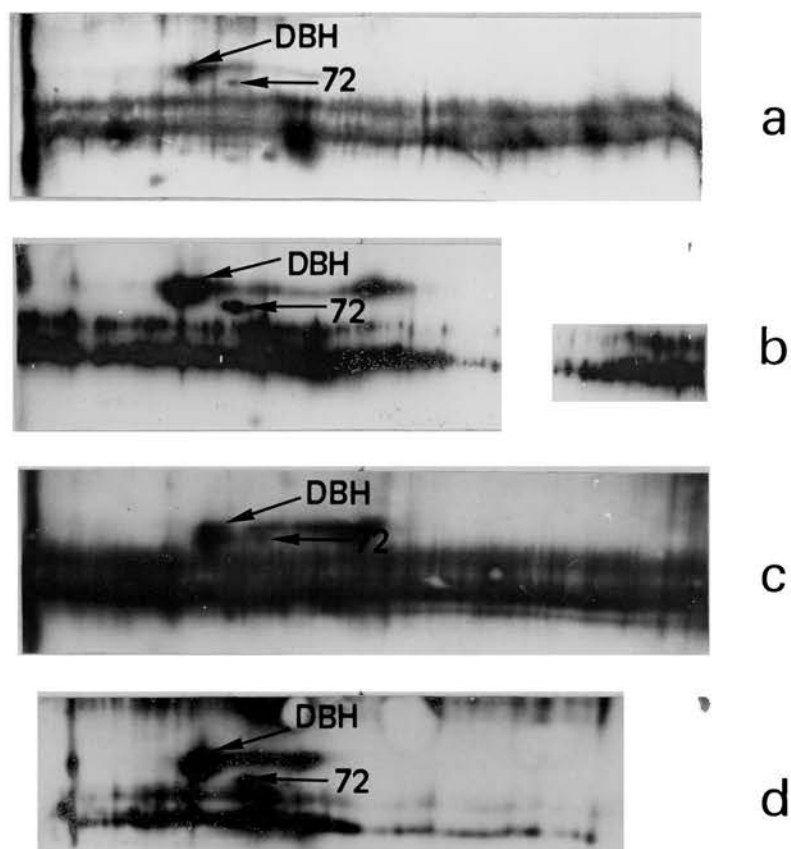


Fig. 5.7. Two-dimensional immunoblots of H^+ -ATPases purified from four bovine sources: (a) chromaffin granules, (b) kidney microsomes, (c) pituitary microsomes, (d) retinal microsomes. The blots shown in Fig. 5.6 were probed with a third primary antiserum, 1:200 dilution of 323 anti-*Kalanchoë* 72 kDa serum. Incubation of the blots with this primary antibody was followed by horse-radish peroxidase conjugated anti-rabbit goat IgG and then visualisation by the ECL system. Figs. 5.7a-d were produced by super-imposing the autoradiograph of the anti-72 kDa blot over that of the DBH blot. In each case only the relevant part of the blot is shown, but there was no significant immunoreactivity elsewhere.

57 kDa subunit of the kidney ATPase may be due to proteolysis or modification of this polypeptide during isoelectric focussing. However, no evidence was found for the existence of any differences between the 72 kDa ATPase subunits found in different bovine tissues (Fig. 5.7).

All the two-dimensional immunoblots were disfigured by two broad streaks of cross-reacting material across the width of the blot. This artefact is believed to be keratin, present as a contaminant in one of the solutions, which has been detected by anti-keratin antibodies present in the antisera. These streaks are particularly prominent in the 72 kDa blots (see Fig. 5.7), indicating that the anti-72 kDa serum contains a large number of anti-keratin antibodies. It was unfortunately not possible to overcome this problem in the time available, but despite the presence of these bands the spots corresponding to the 57 kDa and 72 kDa subunits could be discerned without difficulty.

5.6. Effect of anti 57-kDa and anti 72-kDa antibodies on the proton-translocating activity of the reconstituted tonoplast ATPase

Since the 57 kDa and 72 kDa subunits of vacuolar H⁺-ATPases are respectively proposed to have regulatory and catalytic roles, it was of interest to investigate the possible inhibitory effects of the antibodies directed against these subunits on proton translocation by the reconstituted ATPase. Initially, 10 µl aliquots of the reconstituted enzyme were incubated with 2 µl of crude antiserum at 4°C for 5 min, before being assayed for proton translocation. There was no significant difference in the H⁺-translocation activity of the reconstituted ATPase, between samples

incubated with antiserum or pre-immune serum, although both sera were somewhat inhibitory compared with untreated controls (see Table 5.1). When 10 μ l aliquots of the reconstituted enzyme were incubated with 5 μ l of crude antiserum at 4°C for 1 hour, the resulting H⁺- translocation activity was much lower than that of the control (see Table 5.2). However, this inhibition was also non-specific since pre-immune sera as well as specific antisera produced inhibitory effects.

In an attempt to remove any material from the antisera which might non-specifically interfere with activity of the reconstituted ATPase, purified IgG fractions were prepared from each of the antisera and their corresponding pre-immune sera (see section 5.2 of this chapter). 10 μ l aliquots of the reconstituted enzyme were incubated with 5 μ l of purified IgG at 4°C for 1 hour before being assayed for proton-pumping activity (see Table 5.3). The results reveal that, as with crude antisera, none of the IgG fractions tested have any significant inhibitory effect on the reconstituted ATPase.

Antiserum	H ⁺ -translocation activity (%)
Control	100
320 P.I.	58
320 Anti-72 kDa	69
321 P.I.	71
321 Anti-57 kDa	76
322 P.I.	85
322 Anti-57 kDa	71
323 P.I.	86
323 Anti-72 kDa	81

Table 5.1. Effect of antibodies raised against the 57 kDa and 72 kDa subunits on the H⁺-translocation activity of the reconstituted ATPase. Ten μ l aliquots of the reconstituted enzyme were incubated with 2 μ l of crude antiserum at 4°C for 5 min. All rates are expressed relative to the activity of the control (proteoliposomes not incubated with antiserum). N.B. P.I. = pre-immune serum.

Antiserum	H ⁺ -translocation activity (%)
Control	100
320 P.I.	10
320 Anti-72 kDa	33
321 P.I.	0
321 Anti-57 kDa	19
322 P.I.	16
322 Anti-57 kDa	29
323 P.I.	38
323 Anti-72 kDa	30

Table 5.2. Effect of antibodies raised against the 57 kDa and 72 kDa subunits on the H⁺-translocation activity of the reconstituted ATPase. Ten μ l aliquots of the reconstituted enzyme were incubated with 5 μ l of crude antiserum at 4°C for 1 h. All rates are expressed relative to the activity of the control (proteoliposomes not incubated with antiserum). N.B. P.I. = pre-immune serum.

Antiserum	H ⁺ -translocation activity (%)
Control	100
320 P.I.	76
320 Anti-72 kDa	76
321 P.I.	88
321 Anti-57 kDa	109
322 P.I.	59
322 Anti-57 kDa	47
323 P.I.	57
323 Anti-72 kDa	141

Table 5.3. Effect of antibodies raised against the 57 kDa and 72 kDa subunits on the H⁺-translocation activity of the reconstituted ATPase. Ten μ l aliquots of the reconstituted enzyme were incubated with 5 μ l of purified IgG at 4°C for 1 h. All rates are expressed relative to the activity of the control (proteoliposomes not incubated with antiserum). N.B. P.I. = pre-immune serum.

5.7. Discussion

The cross-reactivity of antibodies raised to selected V-type ATPase subunits from one source with polypeptides of similar size from V-type ATPases from widely different sources is well documented (Bowman *et al.*, 1986; Mandala & Taiz, 1986; Moriyama & Nelson, 1989*b,c*; Manolson *et al.* 1989). It was therefore unsurprising to find that antibodies raised to the 57 kDa and 72 kDa subunits of the *Kalanchoë* tonoplast ATPase cross-reacted with the corresponding subunits of V-type ATPases from a variety of sources (Fig. 5.3). This immunological cross-reactivity is particularly striking since the H⁺-ATPase preparations were not only from different eukaryotic species, but also from different endomembrane organelles.

When the same group of vacuolar ATPases were immunoblotted with antibodies raised against the 120 kDa subunit of the bovine chromaffin granule ATPase, a polypeptide of similar molecular mass present in *Kalanchoë* tonoplast membranes also appeared to cross-react with the 120 kDa antibodies (see Fig. 5.4). This result appears to contradict the data on the subunit composition of the purified ATPase (see Chapter 3, section 3.4), which showed no evidence for a subunit of this size in the *Kalanchoë* enzyme, either by gel staining or by immune blotting. Furthermore, when the subunit composition of the purified tonoplast ATPase from oat roots was reported recently (Ward & Sze, 1992), there was no evidence for a large (approx. 100 kDa) subunit. Therefore the weight of evidence suggests that a 100 kDa subunit is not an essential component of the tonoplast ATPase.

These immunological studies show that vacuolar H⁺-ATPases from a variety of endomembrane organelles and different eukaryotic kingdoms are very closely related to one another and are probably derived from a common

evolutionary ancestor. Immunological cross-reactivity studies have also established that the V-type ATPases are close relatives of the archaeobacterial plasma membrane ATPases and are to a lesser degree related to the F-type ATPases (Konishi *et al.* 1990).

Despite their common features, the endomembrane H⁺-ATPases of different organelles may not be identical, since they appear to differ in physiological properties such as Cl⁻ stimulation and NO₃⁻ inhibition (Cidon *et al.*, 1983; Chanson and Taiz 1985; Ali and Akazawa, 1986), as well as in the ability to use GTP instead of ATP as the energy source for H⁺-translocation (Glickman *et al.*, 1983; Gluck and Al-Awqati, 1984; Rees-Jones and Al-Awqati, 1984; Chanson and Taiz 1985; Ali and Akazawa, 1986). There is also likely to be a need for independent regulation in different organelles. Such regulation, as well as targeting to different organelles, may require differences in some subunits.

When partially-purified ATPase fractions from four different bovine sources were immunoblotted against antibodies raised to the 57 kDa subunit of the *Kalanchoë* tonoplast ATPase, it was revealed that the immunoreactive polypeptide of the kidney ATPase was slightly larger than the equivalent subunit in either the chromaffin granule, pituitary or retinal enzymes (Fig. 5.5a) and also appeared as doublet. This result suggested that there may be isoforms of the bovine V-type ATPase, present in different tissues of the animal.

To investigate further the possible existence of isoforms of the bovine V-type ATPase, purified ATPase fractions from chromaffin granules, kidney microsomes, pituitary microsomes and retinal membranes were analysed by two-dimensional immunoblotting. When the 2-D blots were probed with antibodies directed against the 57 kDa subunit of the *Kalanchoë* ATPase,

the immunoreactive polypeptide of the kidney ATPase was found to show more distinct polymorphism and to have a higher pI than the 57 kDa subunits from the other three ATPases (Fig. 5.6). This suggests that in the kidney distinct isoforms of this subunit are expressed, whereas in the adrenal, pituitary and retina a single isoform, different from that in kidney is expressed. The adrenal medulla, pituitary and retina all contain neurosecretory vesicles which may therefore contain the same type of H⁺-ATPase, whereas in the kidney the function and location of the enzyme are entirely different. Moreover, when the same blots were probed with antibodies directed against the 72 kDa subunit of the *Kalanchoë* ATPase, there was no evidence for the existence of any differences between the 72 kDa ATPase subunits found in different bovine tissues (Fig. 5.7).

It has previously been reported that the vacuolar H⁺-ATPase isolated from bovine kidney brush border differed both enzymatically and structurally from the kidney microsomal H⁺-ATPase (Wang & Gluck, 1990). Differences in the structure of both the 56 kDa and 31 kDa subunits were observed on two-dimensional SDS-polyacrylamide gels of the H⁺-ATPases purified from the two different membrane preparations. A more recent paper has provided further evidence of heterogeneity in the structure of the 31 kDa subunit of the bovine kidney vacuolar H⁺-ATPase (Hemken *et al.*, 1992). Two-dimensional immunoblotting revealed multiple isoforms of the 31 kDa subunit from the brush border H⁺-ATPase, whereas the microsomal H⁺-ATPase showed only a single 31 kDa polypeptide. This study also showed that the membrane distribution of the vacuolar ATPase within the kidney was dependent upon which isoform of the 31 kDa subunit the enzyme possessed. Another recent publication has reported the isolation of two forms of the bovine brain V-type

ATPase 58 kDa subunit from a bovine brain cDNA library (Puopolo *et al.*, 1992).

The structural heterogeneity of specific subunits of the vacuolar ATPase, not only from different tissues but also different membrane fractions within the same tissue may reflect a mechanism for distributing and controlling vacuolar ATPases in many different organelles.

The antibodies directed against the 57 kDa and 72 kDa subunits of the *Kalanchoë* tonoplast ATPase were found not to have any significant inhibitory effect on the H⁺-translocation activity of the reconstituted enzyme. This was not a surprising result since the antibodies were raised to SDS-denatured polypeptides as opposed to structurally intact subunits. Therefore, the epitopes which these antibodies recognise are probably hidden in the native conformation of these polypeptides.

CHAPTER 6

6. General conclusions

6.1. Rapid purification and reconstitution of a plant vacuolar ATPase using Triton X-114 fractionation

A rapid procedure has been developed for the purification and reconstitution into proteoliposomes of the tonoplast H^+ -translocating ATPase from the CAM plant *Kalanchoë daigremontiana*. It involves the fractionation of crude tonoplast membranes with Triton X-114, resolubilization of the ATPase with octyl glucoside in the presence of a mixture of phosphatidylcholine, phosphatidylserine and cholesterol (27:53:20 by weight), and the removal of the detergent by gel filtration. Starting with partially-purified vacuolar membranes, this procedure can be accomplished in about 2 hours and yields vesicles with a high specific ATPase activity-about 3 $\mu\text{mol}/\text{min}$ per mg protein.

This method was adapted from a procedure originally developed for the chromaffin granule H^+ -ATPase (Pérez-Castiñeira & Apps, 1990). It was found to be applicable to tonoplast membranes with some minor modifications, the most important of which was the inclusion of lipids throughout the process of resolubilization, and it is likely that this procedure will be of general applicability in purifying V-type ATPases. Triton X-114 fractionation has been used to produce ATPase-enriched fractions from membranes isolated from several bovine sources (see Chapter 5).

At 2 hours, the Triton X-114 procedure is considerably faster than previously published procedures for the purification of solubilised plant V-type ATPases, which usually involve either glycerol (or sucrose) density gradient centrifugation and/ or column chromatography (Mandala & Taiz,

1985b; Manolson *et al.*, 1985; Randall & Sze, 1986; Bremberger *et al.*, 1988; Matsuura-Endo *et al.*, 1990; Kasamo *et al.*, 1991; Ward & Sze, 1992), and can take up to 40 hours to complete. These established methods typically yield a product of specific ATPase activity 2.0 - 6.0 $\mu\text{mol}/\text{min}$ per mg protein, which is comparable to that obtained with the Triton X-114 method.

Although, a specific activity of 20 - 25 $\mu\text{mol}/\text{min}$ per mg protein has been reported for the tonoplast ATPase of red beet (*Beta vulgaris* L) (Parry *et al.*, 1989). This higher value would imply that the H^+ -ATPase is only a minor component (< 2%) of the tonoplast, which has a specific H^+ -ATPase activity of 0.33 $\mu\text{mol}/\text{min}$ per mg protein; however, polyacrylamide gel electrophoresis suggests that it is a major component (see Chapter 3).

The reconstituted *Kalanchoë* ATPase proteoliposomes undergo ATP-dependent acidification, which can be measured by the quenching of the fluorescence of acridine derivatives. In these studies, 9-aminoacridine was used because it was a more sensitive probe than quinacrine and was not susceptible to ATP-independent fluorescence quenching upon addition of valinomycin, which had been observed with ACMA. The initial rate of fluorescence quenching was shown to be directly proportional to the amount of ATPase protein in the assay and was therefore taken to be directly proportional to the rate of H^+ -translocation. The preparation was thus suitable for studying the kinetics of the tonoplast H^+ -ATPase.

A procedure reported for the purification and reconstitution of the tonoplast H^+ -ATPase from mung bean (Kasamo *et al.*, 1991) yielded proteoliposomes with a rate of H^+ -translocation that was apparently much less than that of vesicles prepared by the Triton X-114 method; in terms of

fractional fluorescence quenching, the rates are 0.0005 and 0.148 per min per μg protein, for the mung bean and *Kalanchoë* enzymes, respectively.

6.2. Subunit composition/ function relationships in V-type ATPases

V-type ATPases are composed of two distinct domains, an integral membrane proton-conducting channel and a peripheral catalytic complex (Forgac, 1989; Nelson, 1991). The exact subunit composition of the holoenzymes is still not known. All contain at least three subunit types, of molecular masses approximately 70, 57 and 17 kDa (see Table 1.1). Additional subunits of 19, 33, 40 and 115-120 kDa have been found in mammalian ATPases (Arai *et al.*, 1987; Moriyama & Nelson, 1987; Apps *et al.*, 1989; Moriyama & Futai, 1990), and comparable compositions have been reported for the V-type ATPases of yeast (Kane *et al.*, 1989) and higher plants (Bremberger *et al.*, 1988; Lai *et al.*, 1988; Parry *et al.*, 1989; Ward & Sze, 1992).

There have been many studies which show that the 70 kDa subunit of V-type ATPases is labelled by the alkylating agents Nbf-Cl and NEM in an ATP-protectable fashion. This strongly suggests that the 70 kDa subunit possesses the catalytic site of the ATPase complex, although it could be argued that the susceptible sulphydryl group is an important regulatory site. Amino-acid sequence similarities between this subunit and the β -subunit of F-type ATPases tend to support the view that it has a catalytic role.

The 57 kDa subunit of the red beet ATPase was also shown to be labelled in an ATP-protectable fashion by the photo-activatable ATP analogue BzATP (Manolson *et al.*, 1985), suggesting that this subunit also possesses a

nucleotide binding site. Without photo-activation, BzATP was shown to be a potent reversible inhibitor of the red beet ATPase. However, BzATP was not a simple competitive inhibitor of the ATPase, but caused the enzyme to display cooperative kinetics with respect to ATP concentration. From these observations, Manolson *et al.*, (1985) concluded that the 57 kDa subunit carried a high affinity ATP-binding site which was essential for catalysis, but which was distinct from the catalytic site, and that this subunit may play a regulatory role. Alternatively, it is conceivable that the 57 kDa (and not the 70 kDa) subunit is in fact the catalytic subunit.

Further investigation of the 70 and 57 kDa subunits is necessary in order to determine the precise role of these subunits. Kinetic experiments (described in section 6.4) may reveal nucleotides that bind exclusively or selectively to either catalytic or regulatory sites. If this is so it would then be possible to attempt affinity labelling of these sites, using radioactive, covalently reacting analogues of these nucleotides. The labelled subunits could then be identified by SDS-gel electrophoresis and autoradiography. Using the results of these experiments it would be possible to relate the current structural model for V-type ATPases (see Fig. 1.7) to the mathematical model developed for the *Kalanchoë* ATPase (see Chapter 4, section 4.4). These experiments would also provide further information on the subunit stoichiometry of V-type ATPases.

No function has yet been assigned to the subunits of intermediate size (20-40 kDa) in V-type ATPases. These subunits are believed to form the "stalk" region which connects the catalytic "head" group to the membrane bound proton channel and so may couple ATP hydrolysis to proton translocation. However, gene-sequencing data for these subunits (Hirsch *et al.*, 1988; Wang *et al.*, 1988; Foury, 1990) has revealed no homology with any known proteins and thus gives no indication of their function.

Most animal V-type ATPases contain a large (100-120 kDa) trans-membrane subunit (Arai, *et al.*, 1987; Moriyama & Nelson, 1987; Apps *et al.*, 1989; Moriyama & Futai 1990; Nelson, 1991; Gillespie *et al.*, 1991), the role of which is as yet unknown. This subunit is glycosylated, the oligosaccharide attachment site presumably being on the luminal side of the membrane.

Removal of the 116 kDa from the coated-vesicle ATPase has been reported to change the specificity of the enzyme for divalent cations, the depleted fragment showing Ca^{2+} -ATPase activity and being unable to support proton-translocation (Xie & Stone, 1988). This remarkable finding has led to speculation that the 116 kDa subunit may play some role in coupling ATP hydrolysis to proton-translocation (Stone *et al.*, 1989).

Although evidence for the presence of a 100 kDa subunit in the vacuolar H^{+} -ATPase of red beet has been reported (Parry *et al.*, 1989), most-groups working on plant vacuolar ATPases have found no evidence for a large subunit of the type found in the mammalian enzymes. The subunit composition of the vacuolar H^{+} -ATPase from oat roots has recently been reported (Ward & Sze, 1992), and the presence of a 100 kDa subunit was not observed in silver-stained gels of the purified ATPase. Similarly, analysis of the purified *Kalanchoë* enzyme by gel staining and immune-blotting failed to reveal the existence of a 100 kDa subunit for this enzyme (see Chapter 3). The reconstituted *Kalanchoë* ATPase is also able to support proton-translocation in the absence of a 100 kDa subunit. Therefore, if the *Kalanchoë* enzyme does possess a 100 kDa subunit, it is not an essential component of the holoenzyme complex, unlike the 116 kDa subunit of the coated vesicle enzyme. So it remains unclear whether a 100-120 kDa polypeptide is an essential component of all V-type ATPases, or is a

species- or a tissue-specific subunit.

6.3. Structural studies of V-type ATPases

There are strong similarities between the structures of V-type ATPases from a wide variety of sources. These enzymes are composed of two distinct domains, a transmembrane proton channel and a peripheral catalytic complex (Forgac, 1989; Nelson, 1991). Dissociation of the ATPase complex is readily achieved in the presence of MgATP under conditions of low temperature and high ionic strength. This 'cold inactivation' treatment has been successfully applied to both plant and animal V-type ATPases and the results from these studies suggest that vacuolar ATPases possess a peripheral complex consisting of five to six different subunit types, including the nucleotide binding 70 and 57 kDa subunits, which is similar to the F_1 domain of F-type ATPases (see Chapter 1 section 1.2.3.4).

The subunit stoichiometry of the ATPase isolated from brain clathrin-coated vesicles has been determined by quantitative amino acid analysis of the separated subunits (Arai *et al.*, 1988). This has led to the formulation of a structural model (see Fig. 1.7) which may be generally applicable to all V-type ATPases. However, this stoichiometry predicts an overall molecular mass of 758 kDa for the ATPase complex (Arai *et al.*, 1988), significantly higher than the usually determined value of 400-600 kDa. Therefore this model must be treated with caution until it can be supported with other types of evidence.

Cross-linking studies can provide important structural information by revealing which subunits are in close proximity in the holoenzyme complex. Such studies have already been carried out on the purified coated-vesicle

H⁺-ATPase, using the cleavable, bifunctional amino reagent 3,3'-dithiobis (sulfosuccinimidylpropionate) (Adachi *et al.*, 1990). These studies indicated that contact regions existed between the 73- and 58-kDa subunits as well as between the 17-kDa subunit and the 40-, 34-, and 33-kDa subunits. If cross-linking experiments were carried out on V-type ATPases from a variety of sources a more refined structural model could be proposed for this class of enzymes.

6.4. Kinetic and regulatory properties of the tonoplast ATPase from *Kalanchoë daigremontiana*

The kinetics of ATP-dependent H⁺-translocation were studied by measuring the initial rates of 9-aminoacridine fluorescence, which is proportional to the net H⁺-flux into the vesicles (Bennett & Spanswick, 1983). 9-aminoacridine was found to be a more suitable probe than 9-amino-6-chloro-2-methoxyacridine (ACMA), which had been used in studying the reconstituted ATPase from chromaffin granules (Pérez-Castiñeira & Apps, 1990), as it was not subject to instantaneous fluorescence changes on addition of valinomycin, Mg²⁺ or ATP. The difference between the two systems is presumably due to different lipid compositions, which were dictated by the requirement of the enzymes.

In the absence of any inhibitors and in the presence of 1 mM free Mg²⁺, the dependence of the rate of H⁺-translocation on the concentration of MgATP²⁻ was adequately described by Michaelis equation with $K_m = 33 \mu\text{M}$. Investigation of the substrate specificity revealed that only purine nucleoside triphosphates (ATP, GTP, ITP) could function substrates for the ATPase and

that only Mn^{2+} and Ca^{2+} could substitute for Mg^{2+} in the complex of ATP with a divalent cation that is the true substrate of the enzyme.

In contrast to the rate of H^+ -translocation, the rate of ATP hydrolysis displayed complex kinetics, which produced non-linear Hanes plots (Fig. 4.1b). The data were best fitted by summing two Michaelis functions which would indicate the existence of two independent catalytic sites with different K_m values, both contributing to the measured rate. The data were also fitted by the Hill equation, with $n_h = 0.55$, suggesting that negative cooperativity occurs between ATP-binding sites. However, the fit was significantly worse than with the two- K_m model.

The investigation of the regulatory properties of the ATPase concentrated on H^+ -translocation because this is the physiological function of the enzyme and because initial rates could be measured reliably. In the absence of inhibitors the enzyme displayed a near Michaelis-Menten dependence on $[MgATP^{2-}]$, with some deviations at very low substrate concentrations ($< 10 \mu M$). However, in the presence of nucleoside diphosphates the ATPase displayed positive cooperativity with regard to substrate binding. This allosteric inhibition was most marked with ADP.

The inhibitory effects of ADP were investigated over a range of ADP concentrations. However, calculation of the Hill parameters for these data gave $n_h > 1$ in the absence of ADP and this value was unaffected by subsequent increases in ADP concentration. This contrasted with findings from a similar study on the chromaffin granule ATPase in which the value n_h increased with ADP concentration (Pérez-Castiñeira, 1991), as would be expected with an allosteric inhibitor. Initially, it was believed that the conflicting results obtained with these two enzymes was due to the smaller substrate concentration range over which the *Kalanchoë* ATPase was

assayed, which may have affected the final values obtained for n_h when the Hill parameters were calculated. Therefore, the inhibition of the ATPase with ADP was re-examined over a larger substrate concentration range, including [MgATP] down to 1.5 μ M. Calculation of the Hill parameters for this second ADP inhibition data set also gave $n_h > 1$ in the absence of ADP and again the value of n_h was largely unaffected by subsequent increases in ADP concentration. Thus, it appears that the fitting of ADP inhibition data to the Hill equation obscures the trend of an increasing value for n_h with increasing ADP concentration. The experimental data from the ADP inhibition study were well fitted by a version of the MWC model in which ATP binds 2 to 3 catalytic sites, with different affinities in the R and T states, and ADP binds to a single regulatory site, in the T state only.

The kinetic studies on the reconstituted *Kalanchoë* ATPase have produced two contradictory findings: the data for ATP hydrolysis suggest that there is negative cooperativity between catalytic ATP-binding sites, whereas the data for ADP inhibition of H^+ -translocation suggests that there is positive cooperativity. At present it is impossible to exclude the possibility that the low- K_m ATP-hydrolytic site is due to a modified or partially dissociated form of the enzyme that does not pump protons. In any case this apparent discrepancy will require further investigation which should lead to a better understanding of the kinetic and regulatory properties of the enzyme. This would involve more extensive inhibition studies as well as nucleotide-binding studies.

Firstly, the inhibition of ATP hydrolysis by nucleoside diphosphates will need to be studied, to see whether this is entirely consistent with the kinetics of H^+ -translocation. For this the coupled assay will be unsuitable, but the enzyme could be assayed by measuring the release of $^{32}P_i$ from

[γ - ^{32}P]-ATP. The inhibition of the enzyme by other nucleotides will also need to be studied to try to find inhibitors that bind selectively to catalytic or regulatory sites, and which might therefore be used in the design of affinity labels for the different sites. Possible candidates for such studies include BzATP, which has already been shown to inhibit the red beet tonoplast ATPase by binding selectively to the 57 kDa subunit (Manolson *et al.*, 1985), and fluorosulphonylbenzoyladenine, which reacts with the 28 kDa subunit of the V-type ATPase from the midgut of larval *Manduca* (Zheng *et al.*, 1992).

Independent estimates of the nucleotide dissociation constants can be obtained by studying the binding of radiolabelled nucleotides to the purified ATPase complex. This should be possible by passage of the holoenzyme through small exclusion columns equilibrated with different concentrations of radio-nucleotides, and determination of the distribution of radioactivity in fractions of the eluate. The results could then be correlated with those obtained in kinetic studies (described above) and in affinity labelling (see section 6.2).

6.5. Evolutionary relationship of the vacuolar H⁺-ATPase to other H⁺-ATPases

Antibodies raised to the 57 kDa and 72 kDa subunits of the *Kalanchoë* tonoplast ATPase were found to cross-react with the corresponding subunits of V-type ATPases from a wide variety of sources. Similar immunological cross-reactivity has been widely reported in the literature (Bowman *et al.*, 1986; Mandala & Taiz, 1986; Moriyama & Nelson, 1989*b,c*; Manolson *et al.*, 1989). These immunological studies show that vacuolar H⁺-ATPases from a

diverse range of endomembrane organelles and different eukaryotic kingdoms are very closely related to one another and are probably derived from a common evolutionary ancestor. Furthermore, immunological cross-reactivity studies have also established that the V-type ATPases are close relatives of the archaeobacterial plasma membrane ATPases (Konishi *et al.*, 1990) and are to a lesser degree related to the eubacterial and eukaryote F-type ATPases (Manolson *et al.*, 1989; Konishi *et al.*, 1990).

The findings from the immunological studies have been supported by gene sequencing information (Gogarten *et al.*, 1989). The amino-acid sequences of both the 70 kDa and 57 kDa subunits are highly conserved. There is 62% sequence homology between the 70 kDa subunits of plants and fungi, while there is over 70% identity between 57 kDa subunits of *Neurospora*, yeast and *Arabidopsis*. When the amino-acid sequences of these 70 kDa and 57 kDa V-type ATPase subunits were compared with those of the corresponding α and β subunits of the archaeobacterial ATPase from *Sulfolobus acidocaldarius*, they were found to be \approx 50% identical. However, the overall identity of the same 70 and 57 kDa V-type ATPase subunits with the corresponding β and α subunits of F-type ATPases is relatively low, about 25%.

From the immunological and gene sequencing data it has been deduced that the vacuolar ATPase of eukaryotic cells is closely related to the plasma membrane H^+ -ATPase of archaeobacteria. This indicates that eukaryotes and archaeobacteria share a common ancestor and that the vacuolar proton pump may have been internalised from the plasma membrane of the common ancestor by a process resembling phagocytosis. The V-type and F-type ATPases diverged at an earlier date, concomitant with the split between eubacteria and protoeukaryotes (Nelson & Taiz, 1989; Gogarten *et al.*,

1989).

6.6. Heterogeneity in the structure of V-type ATPases

When vacuolar ATPases from four different bovine sources were analysed by two-dimensional immunoblotting, with antibodies directed against the 57 kDa and 72 kDa subunits of the *Kalanchoë* tonoplast ATPase, it was revealed that the 57 kDa subunit of the kidney ATPase was an isoform of the 57 kDa subunit found in the chromaffin granule, pituitary and retinal enzymes. Isoforms of both the 56 kDa and 31 kDa subunits have previously been reported for the kidney ATPase (Wang & Gluck, 1990; Hemken *et al.*, 1992). It has also been shown that the distribution of the kidney ATPase was dependent upon which isoform of the 31 kDa subunit is being expressed (Hemken *et al.*, 1992). Two forms of the 58 kDa subunit of the vacuolar H⁺-ATPase from bovine brain have also been identified (Puopolo *et al.*, 1992).

The structural heterogeneity of specific ATPase subunits may facilitate the targeting and regulation of ATPases in individual organelles, although it might be expected that this would be dictated by the accessory subunits (*eg.* 120 kDa) rather than those with a catalytic or regulatory function. However, differences in these may account for differences in physiological properties such as chloride stimulation and nitrate inhibition observed in ATPases from different organelles (Cidon *et al.*, 1983; Chanson & Taiz, 1985; Ali & Akazawa, 1986).

The leaf mesophyll cells of *Kalanchoë daigremontiana* are a rich source of the vacuolar H⁺-ATPase, since this enzyme constitutes over 30% of the tonoplast protein in this CAM plant (Bremberger *et al.*, 1988; Klink *et al.*,

1990). Fractionation of the tonoplast membrane with Triton X-114 has proved to be an effective method for rapid purification and reconstitution of the H⁺-ATPase. Further studies on the reconstituted enzyme should therefore provide an improved understanding of the molecular structure and function of this V-type ATPase.

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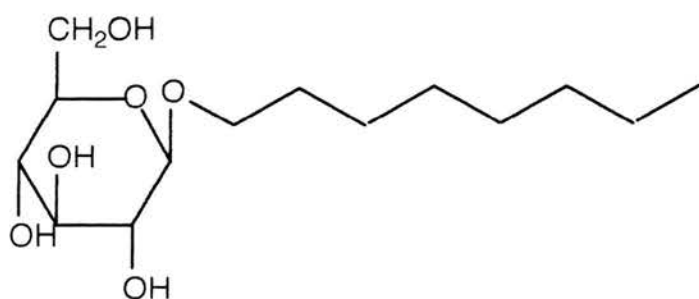
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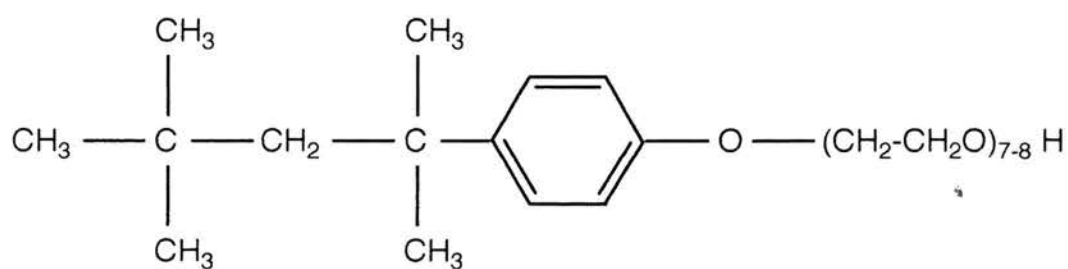
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APPENDIX 1

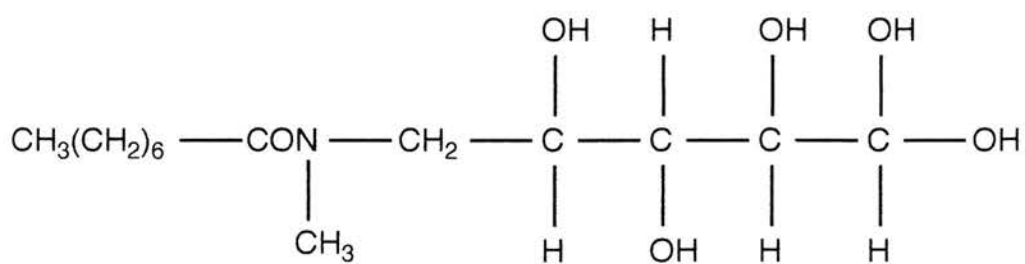
**Structural formulae of some
of the compounds used**



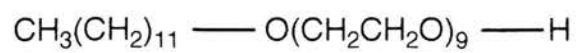
***n*-octyl- β -glucoside (*n*-octyl- β -D-glucopyranoside)**



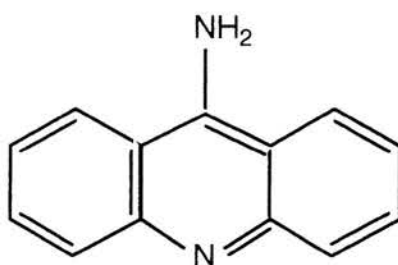
Triton X-114



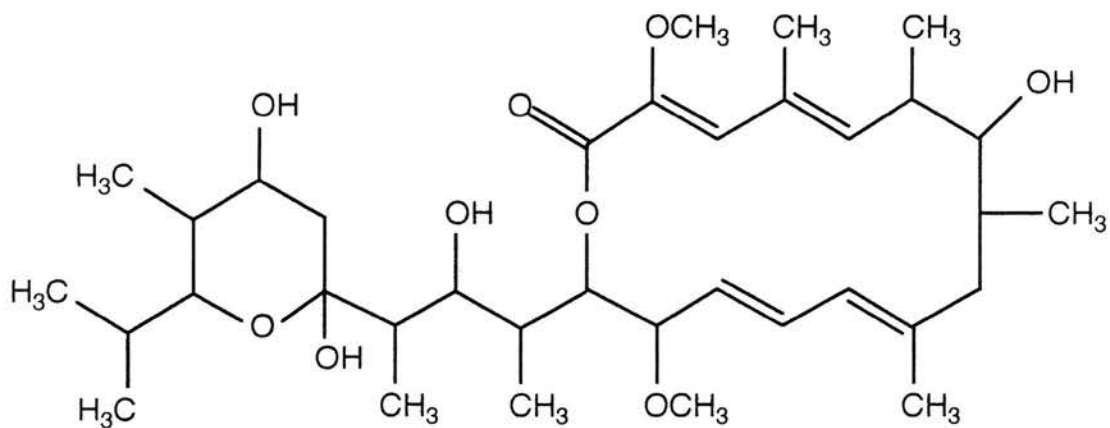
MEGA-8 (octanoyl-*N*-methylglucamide)



C₁₂E₉ (polyoxyethylene 9-lauryl ether)



9-aminoacridine



Bafilomycin A₁

APPENDIX 2

Publications

Reconstitution and kinetics of vacuolar H⁺-translocating ATPases from plants and animals

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Vacuolar (V-type) ATPases constitute one of three major classes of proton-pump, and are multisubunit complexes with structural features in common with F-type ATPases [1]. Although the kinetics of F-type ATPases have been studied in detail, kinetic work on vacuolar ATPases has previously been restricted to measurements of ATP hydrolysis, or of H⁺-translocation by resealed membrane 'ghosts'. We have developed a method for the rapid purification and reconstitution of V-type ATPases, using Triton X-114 to fractionate membranes, and now report kinetic studies on two endomembrane ATPases, one from mammalian secretory vesicles and one from a plant tonoplast.

Proteoliposomes containing the purified H⁺-ATPase from bovine chromaffin granule membranes were prepared as described [2] and the H⁺-ATPase from the tonoplast of *Kalanchoe daigremontiana* by an adaptation of the same procedure [3]. ATP hydrolysis was measured continuously by a spectrophotometric assay, and H⁺-translocation by the quenching of fluorescence of permeant weak bases (9-amino 6-chloro 2-methoxyacridine and 9-amino-acridine). In each case initial rate measurements were made with 1 mM (free) Mg²⁺, and various concentrations of MgATP²⁻. The variances of rate estimates were proportional to the squares of the rates, and kinetic parameters were calculated by fitting various rate equations to experimental data by weighted least-squares regression.

For the purified, reconstituted ATPases from both sources the concentration-dependence of the rate of ATP hydrolysis deviated from Michaelis behaviour. The empirical Hill equation, with $n_H < 1$, could be fitted to the data, but a significantly better fit was obtained with a double Michaelis-Menten function.

The optimized values of the parameters were:

	K_m (μM)	V_{max} (μmol.mg ⁻¹ .min ⁻¹)
Chromaffin granule	1.4 128	0.03 1.28
Tonoplast	1.9 776	0.27 3.65

The Michaelis equation fitted the concentration-dependence of MgATP-driven H⁺-translocation over a 1000-fold range of [MgATP]. However ADP induced apparent cooperativity in saturation with MgATP, together with a reduction in V_{max} . This can be explained by postulating two conformational states of the enzyme (R and T), that differ in their affinities for MgATP and in their catalytic activities [4]. Assuming also that v_0/V_{max} is proportional to the saturation of the enzyme's catalytic sites with MgATP, that ADP binds only to the T-state and that this is inactive, the rate-equation is

$$v_0/V_{max} = \frac{([MgATP]/K_R)(1 + [MgATP]/K_R)^{n-1}}{(1 + [MgATP]/K_R)^n + L'(1 + [MgATP]/K_T)^n} \quad (1)$$

where $L' = L(1 + [ADP]/K_i)^m$, L is the allosteric equilibrium constant ($L = [T]/[R]$) and n and m are the number of binding sites for MgATP and ADP, respectively. Equation (1) was fitted to initial-rate data with [MgATP] and [ADP] varied independently and $m=1$ and $n=2$ or $n=3$ (Fig.1). For both ATPases, setting n to 2 or 3 produced almost equally good fitting of the model, and other values of n and m gave a significantly worse fit. The optimized parameter values were:

	K_R	K_T	K_i	L	Σ
	μM	μM	μM		
Chromaffin granule, $n=2$	29	581	2×10^{-4}	6×10^{-5}	3232
Chromaffin granule, $n=3$	33	205	2×10^{-7}	2×10^{-7}	3689
Tonoplast, $n=2$	3	293	0.95	22	479
Tonoplast, $n=3$	12	142	0.20	1.1	509

(Σ is the sum of squares of residuals)

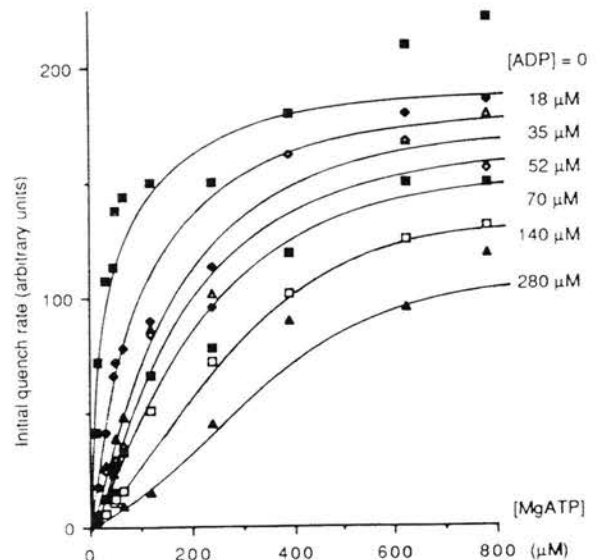


Fig.1 Fitting of data for chromaffin granule ATPase to equation (1)

Figures 1 and 2 show data on the initial rates of H⁺-translocation by chromaffin granule ATPase (Fig.1) and *Kalanchoe* tonoplast ATPase (Fig.2). The lines are predicted by equation (1), with $n=2$, $m=1$. For both ATPases, these data on allosteric effects of ADP are consistent with a two-state concerted transition model [4], with 2 or 3 cooperative binding-sites for MgATP and 1 for ADP. Although this model describes the H⁺-translocation data quite well, it does not account for the ATP-hydrolysis data (see also [5]), which suggest further regulatory features. Furthermore the large number of constants in equation (1) means that their values cannot be determined solely by steady-state kinetics, and separate studies of the number, affinities and specificities of the nucleotide-binding sites are required.

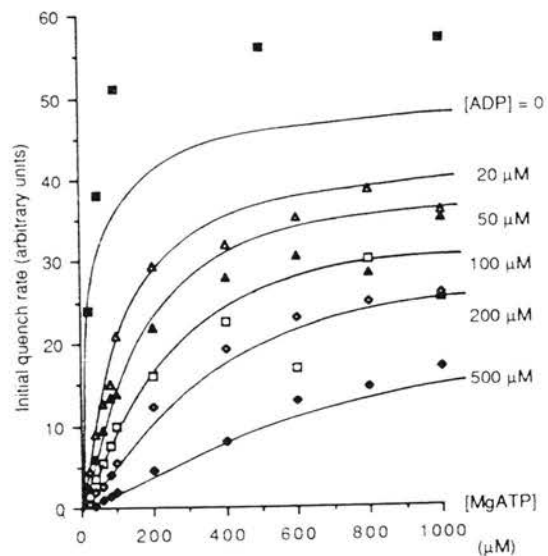


Fig.2 Fitting of data for tonoplast ATPase to equation (1)

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Rapid purification and reconstitution of a plant vacuolar ATPase using Triton X-114 fractionation: subunit composition and substrate kinetics of the H⁺-ATPase from the tonoplast of *Kalanchoë daigremontiana*

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A rapid procedure for the purification and reconstitution into proteoliposomes of the H⁺-translocating ATPase of plant vacuolar membranes is reported. It involves fractionation of the tonoplast with Triton X-114, resolubilization of the ATPase with octyl glucoside in the presence of a mixture of phosphatidylcholine, phosphatidylserine and cholesterol (27:53:20, by weight), and removal of the detergent by gel-filtration. Starting with partially purified vacuolar membranes, the procedure can be accomplished in about 2 hours. It has been applied to the H⁺-ATPase from the crassulacean plant *Kalanchoë daigremontiana*, from which it yields vesicles with a specific ATPase activity of about 3 $\mu\text{mol}/\text{min}$ per mg protein. The purified enzyme contains polypeptides of apparent molecular mass 72, 57, 48, 42, 39, 33 and 16 kDa; these polypeptides also co-sediment on centrifugation of the solubilized ATPase through glycerol gradients. The 16-kDa subunit is labelled with [¹⁴C]dicyclohexylcarbodiimide. There is no evidence for a larger ATPase subunit in this preparation. The reconstituted ATPase proteoliposomes undergo ATP-dependent acidification, which can be measured by quenching of the fluorescence of 9-aminoacridine. The initial rate of fluorescence quenching is a measure of the rate of H⁺ translocation, and is directly proportional to the vesicle protein concentration, so the preparation is suitable for studying the kinetics of the tonoplast H⁺-ATPase. The dependence of the rate of fluorescence quenching on the concentration of MgATP is well fitted by the Michaelis equation, with a K_m value about 30 μM . ATP can be replaced by dATP, ITP, GTP, UTP or CTP, and Mg²⁺ by Mn²⁺ or Ca²⁺; kinetic parameters for these substrates are reported. In contrast, hydrolysis of MgATP shows complex kinetics, suggestive either of negative cooperativity between nucleotide-binding sites, or of two non-interacting catalytic sites. Both the hydrolytic and the H⁺-translocating activities of the proteoliposomes are inhibited by nitrate, though not in parallel, the latter activity being the more sensitive. Both activities are inhibited in parallel by bafilomycin A₁, which does not produce complete inhibition; the bafilomycin-insensitive component has complex ATPase kinetics similar to those of the uninhibited enzyme.

Introduction

Many succulent plants assimilate CO₂ photosynthetically by a process known as crassulacean acid metabolism (CAM) [1,2]. This is characterized by the nocturnal fixation of atmospheric CO₂ into malic acid, which accumulates to high concentrations in the large central vacuoles of photosynthetic cells [3]. The transport of

malate into the vacuole is believed to be energized by two distinct H⁺-translocating enzymes located at the tonoplast, an adenosinetriphosphatase and an inorganic pyrophosphatase [4,5].

The plant tonoplast ATPase is a member of the 'vacuolar' (V-type) class of H⁺-ATPases. These multi-subunit enzymes are associated with the endomembrane systems of eukaryotic cells and include proton pumps present in lysosomes, endosomes, Golgi, secretory vesicles and clathrin-coated vesicles of animal cells, as well as the vacuolar membranes of plants and fungi [6–8]. They hydrolyse cytosolic ATP and translocate protons into the interior of the organelles, thus generating an inside-acid transmembrane pH difference and an inside-positive membrane potential which may be used for secondary processes such as ion transport or solute uptake. Exceptionally, V-type ATPases are found in the plasma membrane [9,10].

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; CAM, crassulacean acid metabolism.

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V-type ATPases are composed of two distinct domains, a transmembrane proton channel and an attached catalytic complex [6,11]. The exact subunit composition of the holoenzymes is not known. All contain at least three subunit types, of molecular masses approximately 70, 57 and 16 kDa, but other subunits may be present. For example, mammalian V-type ATPases have been found to contain additional subunits of 33, 40 and 115–120 kDa [12–15], and comparable compositions have been reported for the V-type ATPases of yeast [16] and higher plants [17–19].

Published procedures for the isolation of plant V-type ATPases involve several steps and typically yield a product of specific activity 2.1–6.0 $\mu\text{mol}/\text{min}$ per mg protein [19–22], although a specific activity of 20–25 $\mu\text{mol}/\text{min}$ per mg protein has been reported for the purified tonoplast ATPase of red beet [17]. Here we describe a rapid method for the partial purification of the tonoplast ATPase from the CAM plant *Kalanchoë daigremontiana* and its reconstitution into proteoliposomes. The method involves the fractionation of tonoplast membranes with the detergent Triton X-114 and resolubilization of the ATPase with *n*-octyl glucoside in the presence of purified lipids. The detergent is then removed by gel filtration, allowing the formation of proteoliposomes. An efficient reconstitution procedure of this kind is an essential prerequisite for detailed study of the kinetics and mechanism of regulation of the enzyme.

Materials and Methods

Materials

Triton X-114 was obtained from Fluka AG, Buchs, Switzerland and purified by the method of Bordier [23], and its concentration determined by the method of Garewal [24]. *n*-Octyl β -glucoside and 9-aminoacridine were purchased from Sigma. Bio-Gel P6-DG was from Bio-Rad. Cholesterol was supplied by Boehringer, Lewes, Sussex, UK. Bovine spinal-cord phosphatidylcholine and phosphatidylserine and egg phosphatidylcholine were obtained from Lipid Products, Redhill, Surrey, UK. Bafilomycin A_1 was a gift from Dr. K. Altendorf, University of Osnabrück, Germany. Antiserum to phosphoenolpyruvate carboxylase from *Kalanchoë daigremontiana* was a gift from Dr. P. Maier and Dr. M. Kluge, Institut für Botanik, Technische Hochschule Darmstadt, Germany. Antiserum directed against the 120-kDa subunit of chromaffin granule H^+ -ATPase was prepared by Ms. J.M. Percy in this Department.

Plant material

Plants of *Kalanchoë daigremontiana* Hamet et Perrier de la Bathie were propagated vegetatively and grown in John Innes No. 3 potting compost in a heated

glasshouse. Natural solar radiation was supplemented by mercury-vapour lamps (400 W MBF; Thorn EMI, London UK) for 12 h daily. When 6–9-month old, plants were transferred to a reverse-phase controlled-environment room, where they were illuminated by a combination of metal-halide fluorescent lamps (400 W MBIF/BU; Thorn) and tungsten lamps (PAR 38 150 W Flood; General Electric Co., Wembley, Middlesex, UK) for 12 h daily at a photosynthetic photon flux density (400–700 nm) of 300 $\mu\text{mol}/\text{m}^2$ per s at mid-plant height. Air temperature was maintained at 25°C (light)/14°C (dark), with a relative humidity of approx. 35% (light)/70% (dark). Plants were maintained in the controlled-environment room for at least 2 days before use.

Tonoplast isolation

Tonoplast fractions were prepared from the mesophyll tissue of *Kalanchoë daigremontiana* according to published methods [5,18] with minor modifications. The leaf midrib and margins were removed and the mesophyll tissue was homogenized at 4°C in a blender in 450 mM mannitol, 10 mM EGTA, 2 mM dithiothreitol, 0.5% (w/v) polyvinylpyrrolidone (PVP-40), 100 mM Tricine, adjusted to pH 8.0 with Tris base; 100 ml medium were used for every 60 g tissue. The homogenate was filtered through two layers of cheesecloth and the filtrate was centrifuged (10000 rev./min, Beckman JA-14 rotor, $g_{av} = 9820$) for 15 min. The resulting supernatant was layered over a 0.78 M sucrose cushion containing 5 mM Tricine-Tris (pH 8.0) and 2 mM dithiothreitol. The gradients were centrifuged (36000 rev./min, Beckman Ti45 rotor, $g_{av} = 100000$) for 1 h. Membrane vesicles were removed from the interface using a Pasteur pipette and diluted 1:1 (v/v) with 150 mM mannitol, 25 mM Tricine-Tris (pH 8.0), 2 mM dithiothreitol. The vesicles were then pelleted by centrifugation (36000 rev./min, Beckman Ti45 rotor) for 30 min, and finally resuspended in 150 mM sucrose, 10 mM Tricine-Tris (pH 8.0), 2 mM dithiothreitol. All fractionation steps were performed at 4°C and the tonoplast preparation was stored at –20°C.

Purification and reconstitution of the H^+ -ATPase

Tonoplast membranes were thawed and sedimented (100000 rev./min, Beckman TL-100.3 rotor, $g_{av} = 340000$, 10 min). All subsequent operations were performed at 0°C. The membrane pellet was resuspended to a final protein concentration of 2.0 mg/ml, using gentle homogenization in a glass homogenizer. The resuspending buffer was 10 mM Hepes/KOH (pH 7.6), 0.15 M KCl, 1 mM EDTA, 2 mM dithiothreitol, containing 6.2 mg/ml Triton X-114. Triton-insoluble material was collected by centrifugation as described above, washed by homogenisation in the same buffer

containing Triton X-114 (6.2 mg/ml), and sedimented again. It was then resuspended in half the original volume of buffer, containing *n*-octyl β -glucoside (68 mM), phosphatidylcholine (2.7 mg/ml), phosphatidylserine (5.3 mg/ml) and cholesterol (2.0 mg/ml); the solution was centrifuged and the clear supernatant carefully removed from the pellet. This supernatant was the solubilized, partially purified ATPase.

Aliquots of 0.2 ml of this solution were loaded onto 1-ml columns of Bio-Gel P6-DG that had been equilibrated with 10 mM Hepes-KOH (pH 7.6), 0.15 M KCl, 2 mM dithiothreitol, 1 mM EDTA, 10% (v/v) methanol, and packed by centrifugation in a bench centrifuge (1400 rev./min; $g_{av} = 180$; 1 min). The column eluates were collected by centrifugation under the same conditions and stored on ice. These proteoliposomes were used for studying ATP-hydrolytic and proton-pumping activities.

Further purification of the tonoplast ATPase resolubilized after Triton X-114 fractionation was performed by centrifugation on glycerol gradients. Gradients (5 ml) were of 5–15% (w/v) glycerol, in 0.15 M KCl, 10 mM Hepes-KOH (pH 7.6), 0.1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) methanol, 35 mM *n*-octyl β -glucoside, 2.7 mg/ml phosphatidylcholine, 5.3 mg/ml phosphatidylserine and 2 mg/ml cholesterol. Aliquots of 0.4 ml of the solubilized ATPase were layered onto these gradients, which were centrifuged for 5 h (Beckman SW50.1 rotor; 45 000 rev./min; $g_{av} = 190\,000$). After centrifugation, the gradients were fractionated into 0.5-ml samples which were assayed for ATPase activity and analysed by SDS-polyacrylamide gel electrophoresis.

¹⁴C]DCCD labelling of tonoplast ATPase

Tonoplast membranes (2 mg/ml), reconstituted ATPase vesicles (0.5 mg/ml) and the fractions from the peak of ATPase activity on glycerol gradients were labelled with [¹⁴C]DCCD (1.85 TBq/ μ mol) by incubation of 100 μ l aliquots of the samples with 11 kBq of [¹⁴C]DCCD for 3 h at room temperature. After separa-

tion by electrophoresis, fluorography of the gels was carried out using 1 M sodium salicylate.

Analytical methods

ATP hydrolysis was measured at 37°C by a coupled spectrophotometric assay described previously [25]. H⁺ translocation by reconstituted ATPase proteoliposomes was measured at 30°C by recording the quenching of 9-amino-acridine fluorescence in a Perkin-Elmer 3000 fluorimeter, with excitation and emission wavelengths of 420 and 480 nm, respectively. The standard assay medium (0.5 ml) contained 0.3 M sucrose, 10 mM Hepes/NaOH buffer (pH 7.4), 1 mM ATP, 1 mM MgSO₄, 9-aminoacridine (1.0 μ M), valinomycin (0.36 μ M) and reconstituted ATPase (2 μ g protein). The initial rate of fluorescence quenching was shown to be directly proportional to the amount of ATPase protein in the assay (data not shown) and was taken to be directly proportional to the rate of H⁺ translocation (an assumption that has also been justified on theoretical grounds [26]). Initial rate data were fitted to the appropriate rate equations by non-linear regression analysis using a computer program written in C programming language and run on the Sequent computer of Edinburgh University Computing Service. The variances of initial-rate estimates were found to be proportional to the square of the measured rate (data not shown), so these were appropriately weighted in the data-fitting procedure.

The protein concentration of the tonoplast suspension was determined by the method of Bradford [27]. All other protein concentrations were measured by an adaptation of the Folin-Lowry method [28].

Results

Purification and reconstitution of the tonoplast H⁺-ATPase

The method used in the purification and reconstitution of the tonoplast H⁺-ATPase was adapted from a procedure developed for the chromaffin granule H⁺-

TABLE I

Reconstitution of tonoplast ATPase

The data shown are for a typical preparation; values in parentheses are the means \pm S.D. of specific activities and recoveries obtained in four separate preparations.

Stage	Activity (nmol/min per ml)	[Protein] (mg/ml)	Specific activity (nmol/min per mg)	Recovery (%)
Membranes	1722	2.0	861 (1006 \pm 21)	(100)
Precipitate	1672	1.55	1079 (1449 \pm 494)	72.9 (86.7 \pm 13.0)
Washed precipitate	1371	1.28	1075 (1265 \pm 357)	59.7 (56.8 \pm 7.2)
Solubilised ATPase	568	0.59	963 (1131 \pm 517)	16.5 (17.2 \pm 8.0)
Reconstituted ATPase	1597	0.59	2707 (2897 \pm 542)	46.4 (42.9 \pm 8.9)

ATPase [29]. Starting with native tonoplast membranes, we were able to produce H^+ -pumping vesicles of high specific ATPase activity within 2 h. The lower concentration of Triton X-114 (0.62%, rather than 2.0%) and the conditions used for solubilization of the ATPase in *n*-octyl glucoside were critically important for successful reconstitution of the plant enzyme. When reconstituting the chromaffin granule V-type ATPase [29], the enzyme was solubilized in *n*-octyl glucoside before adding purified lipids. However, this method proved to be unsuitable when applied to the tonoplast ATPase, as this enzyme was rapidly inactivated when solubilized in *n*-octyl glucoside alone. In order to reconstitute the plant enzyme in an active state, it was essential that the solubilization with *n*-octyl glucoside was performed in the presence of lipids already dissolved in the detergent.

The purification and reconstitution of the tonoplast H^+ -ATPase is summarized in Table I, which shows data for a typical preparation and the average specific activities from four preparations. The final specific activity reported in Table I is that obtained on reconstitution with a mixture of phosphatidylcholine, phosphatidylserine and cholesterol, in the ratio 27:53:20 by weight (see Materials and Methods). This mixture was found to be close to the optimum for both the ATP-hydrolytic and proton-pumping activities of the enzyme, although as the two activities had different lipid dependencies (Table II) the standard conditions adopted were a compromise, reflecting both the intrinsic lipid-dependence of the ATPase and the requirement for sealed vesicles in which H^+ translocation could be measured. There was a three-fold stimulation of ATPase activity on reconstitution of the solubilized enzyme into proteoliposomes. Similar stimulation by

TABLE II

Effect of lipid composition (expressed as the fraction by weight of total lipid) on ATP hydrolysis and H^+ translocation by the reconstituted ATPase

All rates are expressed relative to the activity of proteoliposomes made with the standard lipid composition (underlined).

Phosphatidyl- choline	Phosphatidyl- serine	Cholesterol	ATPase activity	H^+ translocation activity
0.50	0.50	0.0	74	0
0.475	0.475	0.05	77	0
0.45	0.45	0.10	88	0
0.425	0.425	0.15	93	105
0.40	0.40	0.20	85	154
0.375	0.375	0.25	83	59
0.80	0.0	0.20	100	0
0.53	0.27	0.20	126	50
0.40	0.40	0.20	91	89
<u>0.27</u>	<u>0.53</u>	<u>0.20</u>	100	100
0.0	0.80	0.20	62	152

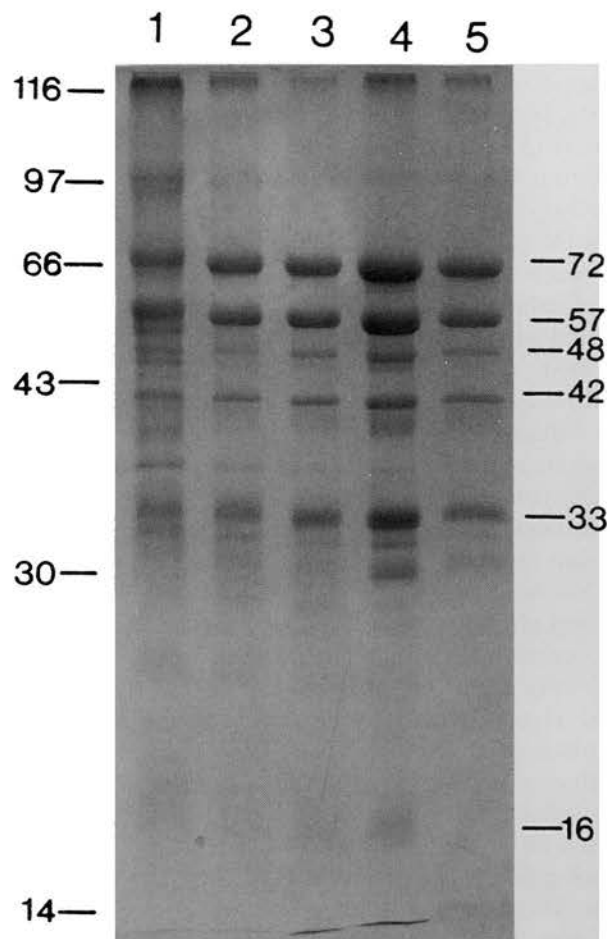


Fig. 1. 12%-polyacrylamide/SDS gel of ATPase fractions at different stages of purification (see Table I), stained with Coomassie blue. Track 1, tonoplast membranes; track 2, protein precipitated with Triton X-114; track 3, washed precipitate; track 4, resolubilized ATPase; track 5, reconstituted ATPase. Each track contained 20 μ g of protein. Figures on the left indicate the positions of molecular mass standards; figures on the right show the apparent molecular masses of major polypeptides (kDa).

lipids was observed with the chromaffin-granule H^+ -ATPase [29], although in this case the optimal lipid composition was quite different.

Subunit composition

Fig. 1 shows a Coomassie blue stained SDS-polyacrylamide gel of the ATPase at various stages of its purification. The reconstituted enzyme contains major polypeptides of apparent molecular mass 72, 57, 48, 42 and 33 kDa; a 16 kDa subunit stains poorly, but is revealed by radioactive labelling (see below). Small amounts of a 100 kDa protein co-purify with the ATPase, but immunoblotting of the fractions shown in Fig. 1 identified this protein as the cytosolic enzyme phosphoenolpyruvate carboxylase (result not shown). Furthermore, antibodies raised against the 120 kDa subunit of bovine chromaffin granule H^+ -ATPase [30]

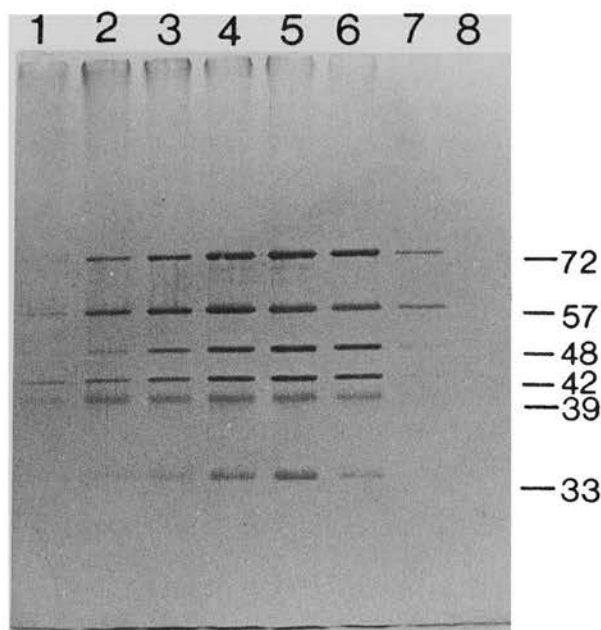


Fig. 2. Subunit composition of the purified ATPase. 10%-polyacrylamide/SDS gel of fractions with ATPase activity on glycerol density-gradient centrifugation, stained with silver. Fraction 4 had the highest activity. Figures on the right of the gel indicate apparent molecular masses (kDa) of subunits.

failed to recognize any antigen in the tonoplast ATPase.

The solubilized ATPase was further purified by centrifugation through glycerol gradients. This procedure removed some minor contaminants from the solubilized ATPase, but the protein concentrations were too low for accurate measurement, so the specific activity could not be determined. A silver-stained polyacrylamide gel of the gradient fractions of highest ATPase activity is shown in Fig. 2. These fractions contain the same polypeptides as the reconstituted enzyme vesicles (Fig. 1); silver-staining also reveals another polypeptide, of apparent molecular mass 39 kDa. The 16 kDa subunit (not seen in Fig. 2, which is a 10%-polyacrylamide gel) was strongly labelled by [^{14}C]DCCD (not shown); some labelling of the 72 and 57 kDa subunits also occurred, although this was not seen when unfractionated tonoplasts were treated with [^{14}C]DCCD.

Kinetic studies of the reconstituted tonoplast ATPase

The reconstituted ATPase vesicles undergo rapid, ATP-dependent acidification, as revealed by the quenching of 9-aminoacridine fluorescence (Fig. 3). This quenching was absolutely dependent on the presence of valinomycin, and was reversed by nigericin or FCCP. The initial rate of quenching of 9-aminoacridine fluorescence was directly proportional to the amount of vesicle protein in the assay, up to the maximum rate that could be measured. The depen-

dence of this rate on the concentration of MgATP^{2-} (in the presence of a fixed concentration of 1 mM free Mg^{2+}) fits a simple Michaelis function, with K_m $33 \pm 1 \mu\text{M}$ (Fig. 4a). However, the kinetics of ATP hydrolysis were more complex, and showed deviation from Michaelis-Menten kinetics (Fig. 4b). The best fit of these data was obtained by assuming two independent catalytic sites, with K_m values of 776 ± 63 and $1.9 \pm 1.2 \mu\text{M}$, and V_{\max} values of 3.65 and $0.27 \mu\text{mol/mg per min}$, respectively. The large S.D. in the lower K_m value is due to the difficulty of measuring reaction rates at very low concentrations of MgATP. Previous studies of ATPase activity in fractions of the native tonoplast from *Kalanchoë daigremontiana* yielded apparent K_m values of $310 \mu\text{M}$ [31] to $810 \mu\text{M}$ [32], depending on the ionic composition of the assay medium.

The substrate specificity of the reconstituted ATPase was also investigated by measuring initial rates of

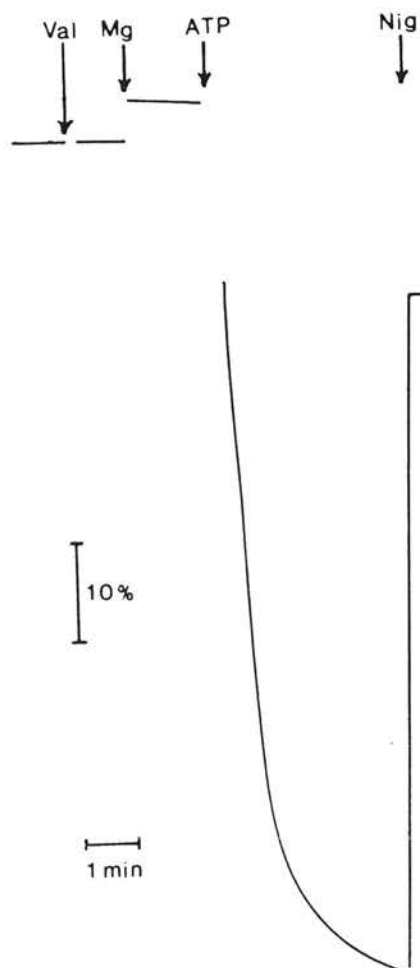


Fig. 3. Quenching of fluorescence of 9-aminoacridine by reconstituted ATPase proteoliposomes. The assay mix (0.5 ml) contained 2 μg of reconstituted ATPase protein. Additions were: Val, valinomycin ($0.36 \mu\text{M}$); Mg, MgSO_4 (2 mM); ATP, adenosine triphosphate (1 mM); Nig, nigericin ($0.67 \mu\text{M}$).

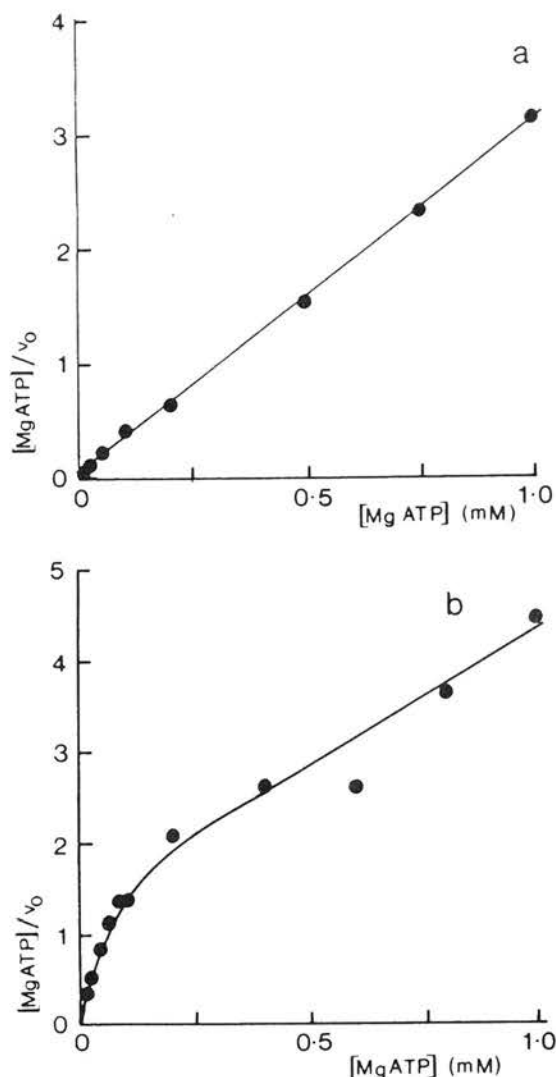


Fig. 4. Kinetics of the reconstituted ATPase. (a) Hanes plot of the dependence of the rate of H^+ translocation on the concentration of MgATP. The data points are experimental (each a mean of four observations). The line is the best fit, calculated for $K_m = 33 \mu M$. Initial rates (v_0) are expressed as fractional fluorescence quench/min per μg protein. (b) Hanes plot of the dependence of the rate of ATP hydrolysis on the concentration of MgATP. The data points are experimental (each a mean of two observations). The line is the best fit, calculated for K_m values of 776 and $1.9 \mu M$. Initial rates (v_0) are expressed as μmol ATP hydrolysed/min per mg protein.

H^+ translocation produced by a range of concentrations of ATP and of other nucleoside triphosphates. The kinetic parameters are shown in Table III. In terms of the relative V_{max} values obtained with the different nucleoside triphosphates (as their magnesium complexes), the relative substrate effectiveness is

$$dATP > ATP > ITP > GTP = UTP \gg CTP$$

With CTP as substrate the activity of the ATPase was so low that the K_m value could not be measured. This result demonstrates that hydrolysis of alternative nu-

cleoside triphosphates, which was observed previously in preparations of isolated vacuoles [31], is a genuine property of the ATPase.

Table III also shows the effect of different divalent cations on the H^+ -translocating ability of the reconstituted ATPase. No H^+ translocation was measurable with Zn^{2+} as the activatory cation; investigation of the effects of Co^{2+} or Ni^{2+} was precluded by their quenching of the fluorescence of 9-aminoacridine.

Inhibition of the reconstituted ATPase by nitrate and bafilomycin

Nitrate is a characteristic inhibitor of V-type ATPases [6]. The inhibition by nitrate of ATP hydrolysis and H^+ translocation is shown in Fig. 5a. Nitrate is a noncompetitive inhibitor of H^+ translocation, the K_m for $MgATP^{2-}$ being unaltered by concentrations of NO_3^- up to 50 mM (data not shown). As with native membranes from *Kalanchoë* [32], H^+ translocation is more sensitive to nitrate inhibition than is ATP hydrolysis, the inhibitor concentrations producing half-maximal inhibition of the reconstituted enzyme being 4 and 9 mM, respectively.

A more specific and potent inhibitor of V-type ATPases is bafilomycin A_1 [33], which inhibits ATP hydrolysis and H^+ translocation in parallel (Fig. 5b). The apparent I_{50} value in this experiment (0.18 ng bafilomycin/ μg protein) extrapolates to saturation at approximately 0.3 mol inhibitor/mol ATPase (assuming that the enzyme is pure and has a molecular mass of about 500 kDa [18]). This suggests that bafilomycin binds with very high affinity, and effectively titrates out the enzyme, so that the I_{50} value is dependent on the protein concentration. Even at high concentrations of bafilomycin, there is a residual activity (approx. 10% of the uninhibited rate) which is resistant to this inhibitor.

TABLE III

Kinetic parameters of the reconstituted ATPase

Initial rates of H^+ translocation were determined by the quenching of 9-aminoacridine fluorescence, and fitted to the Michaelis equation. Because the rates were measured in arbitrary units, the quoted values of V_{max} are relative to those with MgATP. Each data set contained 28 rate measurements, over a 100-fold range of substrate concentration (cf. Fig. 3a). n.d., not determined.

Substrate (μM)	K_m (%)	Relative V_{max}
MgATP	33 ± 1	100
MgdATP	17 ± 1	204
MgCTP	n.d.	4
MgGTP	86 ± 4	56
MgITP	162 ± 5	70
MgUTP	263 ± 9	46
CaATP	24 ± 1	44
MnATP	10 ± 0.3	111

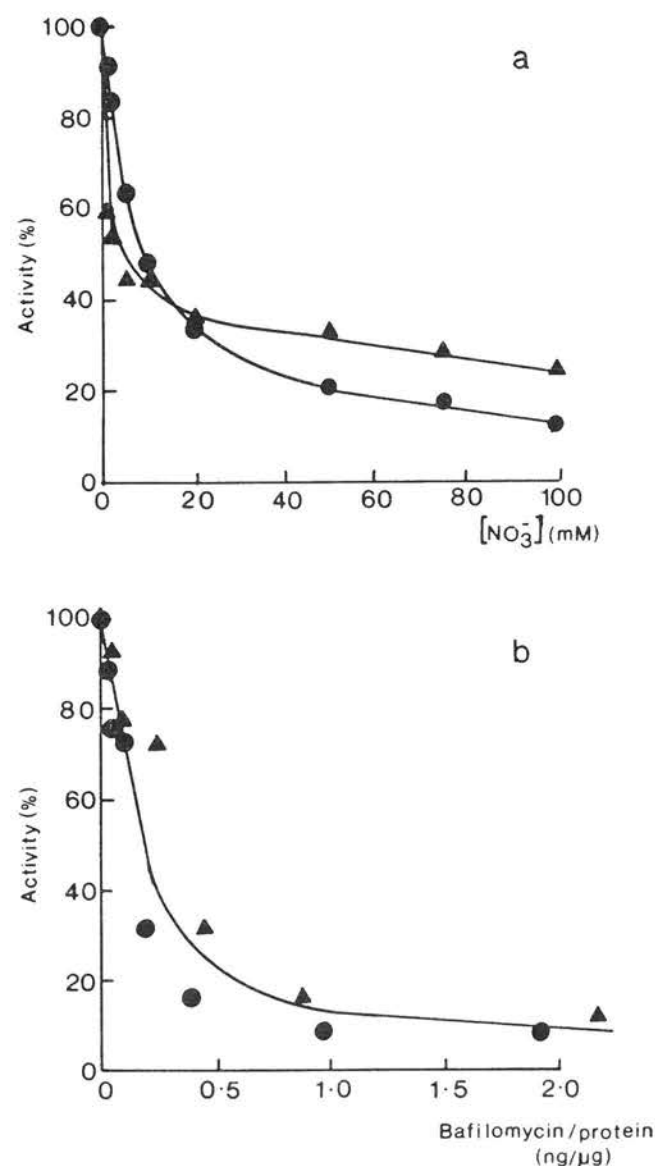


Fig. 5. Inhibition of the reconstituted ATPase. (a) Inhibition by nitrate. (b) Inhibition by bafilomycin A_1 . \blacktriangle , H^+ translocation; \bullet , ATP hydrolysis.

However, this activity was found to be nitrate-sensitive and to display the complex kinetics of ATP hydrolysis described above.

Discussion

Although considerable progress has been made in defining the structures of V-type H^+ -ATPases, rather few kinetic studies have so far been reported. The procedure for ATPase purification and reconstitution, involving the fractionation of membranes with Triton X-114, resolubilization with octyl glucoside and exchange of detergent for lipid by gel-exclusion chromatography, was originally developed for the enzyme

from bovine chromaffin-granule membranes [29]. It was found to be applicable to plant tonoplast with some modifications, the most important of which was the inclusion of lipids throughout the process of resolubilization. It is likely that this procedure will be of general applicability in purifying V-type ATPases; it can be completed within 2 hours, and yields ATPase proteoliposomes of high specific activity and good stability, suitable for kinetic studies. A procedure for reconstitution of the tonoplast H^+ -ATPase from mung bean has recently been published [34]. Although the specific activity of this preparation was not reported, the rate of H^+ translocation was apparently much less than in the present work (compare Fig. 3 with Ref. 34, Fig. 2); in terms of fractional fluorescence quenching, the rates are 0.37 and 0.0005 min^{-1} per μg protein, respectively.

Tonoplast preparations from the CAM plant *Kalanchoë daigremontiana*, from which purification of the vacuolar (V-type) ATPase is reported in this paper, are a particularly good source of the enzyme, as judged by their high specific ATPase activity and the relative abundance of ATPase subunits in the unfractionated membranes (Fig. 1). The purified enzyme contains polypeptides of apparent molecular mass 72, 57, 48, 42, 39, 33 and 16 kDa, and these co-sediment during glycerol-gradient centrifugation. This subunit composition is very similar to that determined by Bremberger et al. [18] for *Kalanchoë daigremontiana*, except that the present work revealed an additional polypeptide of 39 kDa. Although a large (approx. 100 kDa) glycosylated subunit is a component of most, if not all animal V-type ATPases [11–15,30] the function of such a subunit remains unknown. We found no evidence for a subunit of this size in the *Kalanchoë* enzyme, either by gel staining or by immune blotting, although small amounts of phosphoenolpyruvate carboxylase (one of the major leaf proteins in CAM plants) co-purified with the ATPase. However, the presence of a 100 kDa subunit in vacuolar H^+ -ATPase of red beet has been reported [17], so it is still unclear whether a polypeptide of 100–120 kDa is a necessary component of all V-type ATPases, or is a species- or tissue-specific subunit.

The kinetics of ATP-dependent H^+ translocation were studied by measuring the initial rates of quenching of 9-aminoacridine fluorescence, which is proportional to the net H^+ -flux into the vesicles [26]. In studying the reconstituted ATPase from chromaffin granules [29], 9-amino-6-chloro-2-methoxyacridine (ACMA) was used as a probe, but in the present work 9-aminoacridine was found to be more suitable, as it was not subject to instantaneous fluorescence changes on the addition of valinomycin, Mg^{2+} or ATP (Fig. 3). The difference between the two systems is presumably due to the different lipid compositions, which were

dictated by the requirements of the enzymes (compare the data in Table II with those reported in Ref. 29).

In the absence of ADP and the presence of 1 mM free Mg^{2+} , the dependence of the rate of H^+ translocation on the concentration of $MgATP^{2-}$ was adequately described by the Michaelis equation, with $K_m = 33 \mu M$ (Fig. 4a). Similar kinetics were found with other purine nucleotide triphosphates, or when Mg^{2+} was replaced with other divalent cations (Table II). The rate of ATP hydrolysis, however, showed complex kinetics, with nonlinear Hanes plots (Fig. 4b). What is the basis of this unusual substrate dependence, and why does it not apply to H^+ translocation too? In principle, plots of this type would be expected if two or more catalytic centres with different K_m values contribute to the measured rate; or alternatively if there is negative cooperativity between nucleotide-binding sites. Hanada et al. [36] reported similar kinetic behaviour in a study on the chromaffin granule membrane ATPase, and determined three separate K_m values by graphical procedures. The data shown in Fig. 4b could be satisfactorily fitted by summing two Michaelis functions, but were not better fitted by the sum of three Michaelis functions. Alternatively, the data could be fitted to the empirical Hill function

$$v_o = V_{max} / (1 + K/[S]^{n_h})$$

with $n_h = 0.55$. This low value of the Hill coefficient would suggest negative cooperativity between ATP-binding sites, as occurs in F-type ATPases [37], but the fit was significantly worse than with the two- K_m model. It seems unlikely that the reconstituted preparation is contaminated with other ATPases, as it is insensitive to efrapreptin and vanadate, inhibitors of F-type and P-type ATPases, respectively. It might contain small amounts of a modified or dissociated form of the enzyme, formed perhaps during solubilization with octyl glucoside, that is incompetent in H^+ translocation. However, it is noteworthy that kinetics of this type, as well as being a property of the reconstituted chromaffin granule enzyme [36,38], also apply to ATP hydrolysis by resealed chromaffin granule ghosts (Pérez-Castñeira, J.R. and Apps, D.K., unpublished data), although a further complication in this system is the presence of a second membrane ATPase [39].

It is not clear why the kinetics of ATP hydrolysis and ATP-dependent H^+ translocation are different, but it is noteworthy that the two activities are differentially inhibited by nitrate, and such differential inhibition has been observed elsewhere [40].

Fractionation of the tonoplast membrane with Triton X-114 has proved to be an effective method for rapid purification and reconstitution of the V-type H^+ -ATPase. Leaf mesophyll cells of *Kalanchoë daigremontiana* are a rich source of this enzyme, since the

H^+ -ATPase constitutes over 30% of the tonoplast protein in this CAM plant [18,41]. Further studies on the reconstituted enzyme should therefore provide an improved understanding of the molecular structure and function of this V-type ATPase.

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The vacuolar H⁺-translocating ATPase of renal tubules contains a 115-kDa glycosylated subunit

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Kidney microsomes were fractionated with Triton X-114, to give a fraction enriched in the renal tubule H⁺-translocating ATPase, as judged by the sensitivity of its ATPase activity to bafilomycin A₁, and its content of two polypeptides recognized by antibodies directed against subunits of plant tonoplast ATPases. This fraction contained a polypeptide of apparent molecular mass of 115 kDa, that was recognized by an antibody to the largest (120 kDa) subunit of chromaffin-granule membrane H⁺-ATPase, and, like this subunit, was reduced in molecular weight on treatment with glycopeptidase F. We conclude that, like other mammalian vacuolar H⁺-ATPases, the kidney H⁺-ATPase contains a large, glycosylated subunit.

Kidney; Proton translocation; ATPase; Glycoprotein

1. INTRODUCTION

Three classes of H⁺-translocating ATPase have been recognized [1]: F-type (the ATP-synthases of energy-transducing membranes), P-type (proton pumps, located in the plasma membranes of plant, fungal and some specialized animal cells), and V-type (endomembrane proton pumps). The V-type ATPases have a wide distribution, occurring in the membranes of most (probably all) types of acidic intracellular compartments in eukaryotic cells [2]. In renal tubular cells, ATP-driven proton transport is carried out by V-type ATPases that are inserted into the apical plasma membrane by fusion of exocytotic vesicles, in response to changes in acid/base status [3,4].

V-type ATPases are of high molecular weight (400–600 kDa) and complex subunit composition. There is now a consensus that they have a minimum of 3 subunit types: (i) 66–73 kDa; (ii) 55–62 kDa; and (iii) 13–17 kDa, as judged by SDS-polyacrylamide gel electrophoresis. These subunits have been implicated in ATP hydrolysis, regulatory nucleotide-binding and H⁺-translocation, respectively [5–8]. Subunits of intermediate size (20–40 kDa) are also present in most preparations. In some cases up to 5 polypeptides in this range have been reported, but none has yet been characterized functionally.

An interesting controversy surrounds the largest (100–120 kDa) subunit. This was originally found in just two types of mammalian V-ATPase [9–11], but a

polypeptide of comparable size has now been reported to occur in V-ATPases from beet and yeast vacuoles [12,13], although most groups working with plant or fungal V-type ATPases do not find it in their preparations. A subunit of this size was not found in immunoaffinity-purified ATPase of bovine renal cortex and medulla [14], and it was not detected by immune blotting of liver lysosomal membranes and kidney microsomes [15]. We now report the immunochemical detection of a 115 kDa *N*-glycosylated component in partially-purified kidney microsomal V-ATPase and in the ATPase of chromaffin granules isolated from human pheochromocytoma.

2. MATERIALS AND METHODS

Kidney microsomes were prepared by an adaptation of a published procedure [16]. All solutions contained 10 mM Tris-HCl, 1 mM NaHCO₃, 1 mM EDTA, 1 mM DTT (pH 8.0, 0°C), plus sucrose at the required concentration. Bovine renal medullary tissue was minced and homogenized in 0.25 M sucrose, and centrifuged for 10 min at *g*_{av} = 1600. After centrifugation of the supernatant for 60 min at *g*_{av} = 41 000, crude microsomes were removed from the surface of the pellet by swirling, collected by centrifugation, resuspended in 0.25 M sucrose, overlaid onto step gradients of 0.7, 1.0 and 1.5 M sucrose, and centrifuged for 5 h at 35 000 rpm in a swingout rotor (Beckman SW41). The microsomal fraction was collected from the 0.7 M/1.0 M sucrose interface, diluted, centrifuged and finally resuspended in 0.25 M buffered sucrose containing benzamidin (2 mM), pepstatin (5 µg/ml), leupeptin (5 µg/ml).

Triton X-114 (Fluka) was precondensed as described [17]. It was used to fractionate kidney microsomes essentially as described for chromaffin-granule membranes [18,19], except that the final concentration of detergent was 17.5 mg/ml. The Triton-insoluble fraction was used for immune blotting as described below. Chromaffin granule membranes were prepared from bovine adrenal medulla and human pheochromocytoma as described [20] and fractionated with Triton X-114 [19]. Antiserum to chromaffin granule H⁺-ATPase was

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raised by injecting reconstituted holo-ATPase [18] into rabbits. Rabbit antisera to individual subunits of the tonoplast H^+ -ATPase of *Kalanchoe daigremontiana* were raised by injection of proteins electroeluted from gels. Immune blots were developed using the ECL system (Amersham). Deglycosylation of proteins in the Triton-insoluble fraction was carried out by incubation (8 h, 20°C) of 100 μ g protein with 1 unit endoglycosidase F (Boehringer) in 50 mM HEPES-NaOH, pH 7.0, containing 2 mM benzamidine, 5 μ g/ml pepstatin, 5 μ g/ml leupeptin, 0.2 mM PMSF, 0.5% deoxycholate, 1.0% Triton X-100, 20 mM EDTA and 10 mM dithiothreitol (total volume 105 μ l).

3. RESULTS

The purified kidney microsomes had a specific ATPase activity of 0.37 μ mol/min/mg protein, and this could be inhibited 15% by 2 μ M bafilomycin A_1 , a specific inhibitor of V-type ATPases [21]. We established that the optimal concentration of Triton X-114 for fractionating kidney microsomes was 1.75% (w/v); the Triton-insoluble fraction had only a slightly higher specific ATPase activity than did the membranes (0.48 μ mol/min/mg protein), but it was inhibited about 80% by bafilomycin, indicating an enrichment in vacuolar ATPase.

This enrichment is confirmed by the immune blots shown in Fig. 1. Rabbit antisera directed against the 57 kDa and 67 kDa subunits of the tonoplast H^+ -ATPase of *Kalanchoe daigremontiana* recognize polypeptides of similar molecular weight in bovine chromaffin granule membranes and kidney microsomes, and in each case the immunoreactive polypeptides appear in the Triton-insoluble fraction. The anti-57 kDa serum recognizes a doublet in the kidney fraction (Fig. 1b, track 4); a similar result was obtained with antiserum directed against the 57 kDa subunit of beet tonoplast H^+ -ATPase (not shown).

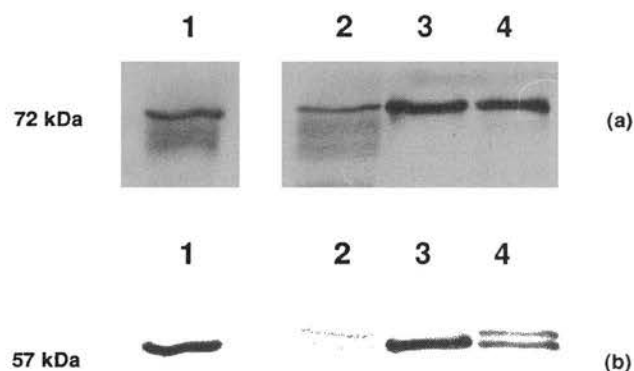


Fig. 1. Immunoblots showing the enrichment in vacuolar ATPase obtained by fractionating membranes with Triton X-114 (Track 1) Chromaffin granule membranes (60 μ g protein). (Track 2) Kidney microsomes (60 μ g). (Track 3) Triton-insoluble fraction from chromaffin granule membranes (10 μ g). (Track 4) Triton-insoluble fraction from kidney microsomes (30 μ g). Antibodies were against the 67 kDa (a) and 57 kDa (b) subunits of a plant tonoplast ATPase; note that in animals these recognize proteins of 72 and 57 kDa, respectively. In each case only the relevant part of the blot is shown, but there is no significant immunoreactivity elsewhere.

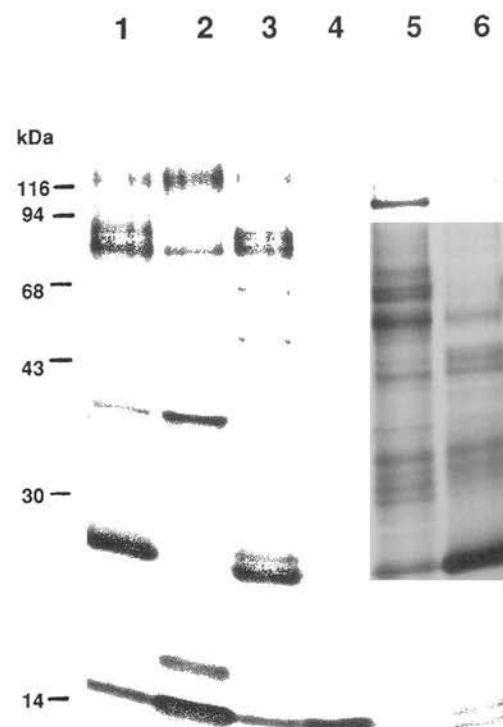


Fig. 2. Silver-stained gel showing unfractionated membranes (tracks 1, 3 and 5) and Triton-insoluble fractions (tracks 2, 4 and 6). (Tracks 1 and 2) Bovine chromaffin granules; (tracks 3 and 4) human phaeochromocytoma granules; (tracks 5 and 6) bovine kidney microsomes. Each track contained 3 μ g protein. The positions of marker proteins are shown at the side; major bands in tracks 2 and 4 are at 120, 72, 57, 40, 19 and 16 kDa.

Although fractionation of chromaffin granule membranes with Triton X-114 purifies the H^+ -ATPase to near-homogeneity [18], the Triton X-114-insoluble fraction from kidney microsomes contains many polypeptides besides those identified as H^+ -ATPase subunits (Fig. 2).

The antiserum produced by immunization of rabbits with reconstituted chromaffin-granule H^+ -ATPase vesicles [18] reacted with the 120 kDa subunit of this ATPase, and (more weakly) with dopamine β -monooxygenase (Fig. 3a, track 1). In unfractionated kidney microsomes, immunoreactivity was barely detectable (track 2), but the Triton X-114-insoluble fraction contained a protein of about 115 kDa that was clearly recognized by this serum (track 4).

Digestion of the Triton X-114-insoluble fraction with endoglycosidase F converted the diffuse immunoreactive band at 115 kDa into a sharper one at about 100 kDa (Fig. 3b, tracks 5 and 6). This is similar to the effect of deglycosylation on the 120 kDa H^+ -ATPase subunit in both bovine and human chromaffin granules (Fig. 3b, and [10]).

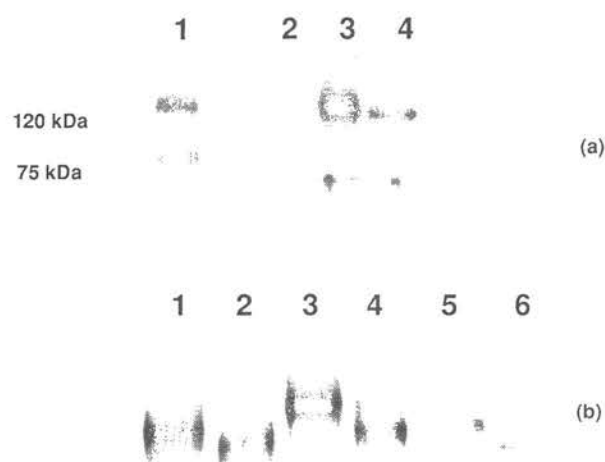


Fig. 3. Immunoblots showing that an antibody to the 120 kDa subunit of chromaffin granule membrane H^+ -ATPase recognizes a similar component in kidney membranes. (a) Fractionation with Triton X-114. (Track 1) Chromaffin granule membranes (20 μ g protein); (track 2) kidney microsomes (20 μ g); (track 3) chromaffin granule membrane Triton-insoluble fraction (5 μ g); (track 4) kidney microsome Triton-insoluble fraction (28 μ g). The immunoreactive band at 75 kDa is dopamine β -monooxygenase. (b) Digestion with endoglycosidase F; Triton-insoluble fractions from: (tracks 1 and 2) human pheochromocytoma granule (5 μ g protein); (tracks 3 and 4) bovine chromaffin granule (5 μ g); (tracks 5 and 6) bovine kidney microsomes (25 μ g). Tracks 1, 3 and 5 are controls; tracks 2, 4 and 6 are endoglycosidase F-treated.

4. DISCUSSION

Fractionation with Triton X-114 [16,18] has proved useful in purifying V-type H^+ -ATPases from chromaffin granule membranes [18] and plant tonoplasts [22], essentially in one step. When applied to kidney microsomes, it produces a Triton-insoluble fraction that is enriched about 5-fold in V-ATPase, as judged by its sensitivity to bafilomycin, but which contains many other polypeptides (Fig. 2). The activity of this preparation can be increased by the addition of phospholipids (cf. [18]) but the conditions have not yet been optimized.

Our attempts to raise antibodies to chromaffin granule membrane H^+ -ATPase by immunization with individual subunits have proved unsuccessful; however, antibodies to the 57 kDa and 67 kDa subunits of plant tonoplast H^+ -ATPases crossreact with the mammalian counterparts [23], and antisera raised against individual subunits of the tonoplast H^+ -ATPase from the crassulacean plant *Kalanchoe daigremontiana* have been used to follow the kidney H^+ -ATPase during Triton X-114 fractionation. Immunoblotting using these antisera confirms the results obtained, using bafilomycin-sensitive ATPase activity as an assay.

Immunization of rabbits with reconstituted chromaffin-granule H^+ -ATPase produced an antiserum that reacted with the largest (120 kDa) subunit.

Its slight reactivity with dopamine β -monooxygenase, a contaminant of the purified H^+ -ATPase, was not a nuisance in these experiments, since this enzyme is absent from kidney; it could in any case be overcome by absorption of the serum with purified dopamine β -monooxygenase, without affecting the reactivity of the serum with the larger component. The antiserum recognizes a polypeptide in kidney microsomes that has an electrophoretic mobility close to that of the 120 kDa subunit of chromaffin-granule H^+ -ATPase, and is glycosylated to a similar extent. In fact, the bovine kidney polypeptide is slightly smaller than that in bovine chromaffin granules, with an apparent molecular weight of about 115 kDa; this is reduced by about 15 kDa on treatment with endoglycosidase F (specific for N-linked oligosaccharide chains). It is established that the 120 kDa subunit is a component of the H^+ -ATPase of chromaffin granules and clathrin-coated vesicles [9–11] and this result suggests that the kidney H^+ -ATPase is similar, this polypeptide having previously been undetected by protein staining and immune blotting. Several of the properties of this subunit make it difficult to detect: it is glycosylated, so appears as a diffuse band in electrophoretograms; it stains poorly with Coomassie blue; it is extremely sensitive to proteolysis; and it aggregates when heated in SDS.

Interestingly, the antibody raised against bovine H^+ -ATPase fails to recognize any antigen in plant tonoplasts [22], although the presence of a 100 kDa subunit in beet H^+ -ATPase has been reported [13]. The question of whether a polypeptide of 100–120 kDa is a necessary component of all V-type ATPases, or is a species- or tissue-specific subunit, remains open.

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